High throughput autoantigen discovery in the spondyloarthropathies and the development of a CFSE based CTL killing assay

by
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ABSTRACT

Since 1974, when Moll and Wright first classified the seronegative spondyloarthropathies on the basis of their lack of positivity for rheumatoid factor, little insight has been made into the role played by the adaptive immune system within these diseases. We know in other rheumatic diseases like rheumatoid arthritis, systemic lupus erythematosus, myositis, and scleroderma that not only are autoantibodies present, but that in each of these diseases multiple specificities have been observed. Additionally, in these rheumatic diseases there is a striking correlation between autoantibody specificity and clinical phenotype. This observance highlights the significant force that B cell mediated autoantibodies play at the molecular level in impacting a patient's course of disease. New technological advancements have led to large scale screens aimed at identifying clinically relevant autoantibodies. In the spondyloarthropathies, a number of groups have claimed to identify a limited set of autoantigens. Here, in chapters 1 and 2, we used the most advanced of these screens by interrogating a human protein array (HuProt array) consisting of ~17,000 recombinantly expressed human proteins with patient serum from psoriatic arthritis and ankylosing spondylitis. This high throughput, exploratory approach led to the identification of TBX20 and IFN-α as autoantigens in a cohort of psoriatic arthritis patients.

In chapters 3 and 4, we lay the groundwork for the development of a scQa-1^b; β2m -/- mouse model to explore Qa-1 dependent T cell development and peripheral CD8+ T cell phenotype. Predominantly, our knowledge of T cell development is centered on the role played by classical MHC Ia molecules. However, far less is known about nonclassical MHC Ib development. Previous work by others, has led to insights suggesting MHC Ib mediated selection can lead to peripheral CD8+ T cells with an "innate-like" phenotype, while other groups have suggested the presence of a Qa-1 restricted CD8+ regulatory T cell lineage. Using this mouse model we aimed to provide additional support and insights into the presence of these T cell subsets. However, as we will discuss in detail

while this transgene did express in a number of cell lines, our mouse model failed to demonstrate *in vivo* scQa-1^b expression.

During this process we also developed a CFSE based CTL killing assay, that provides numerous advantages over the commonly used ^{51}Cr release assay.

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TABLE OF CONTENTS

LISTS OF TABLES

LIST OF FIGURES

CHAPTER I –INTRODUCTION: ANKYLOSING SPONDYLITIS AND PSORIATIC ARTHRITIS

INTRODUCTION: ANKYLOSING SPONDYLITIS AND PSORIATIC ARTHRITIS

Background: The spondyloarthropathies

The spondyloarthropathies (SpA) are a subset of rheumatic diseases that consists of five clinically distinct diseases with overlapping phenotypes as presented by patients. These five diseases consist of ankylosying spondylitis (AS), psoriatic arthritis (PsA), inflammatory bowel disease associated arthritis, reactive arthritis, and undifferentiated spondyloarthritis.[1,2] It was Moll and colleagues, in 1974, who defined the criteria for a subset of seronegative spondarthritides. In this seminal, clinical paper they outlined six criteria to define the disease, as shown in Table 1.1.[3] Highlighted in the nomenclature were the use of the prefix "spond-" and seronegative. "Spond-" referring to the vertebral involvement, and the association these diseases have with the hallmark phenotype of AS. The seronegative title identifies these patients as testing negative for rheumatoid factor (RF). RF is a serological, autoantibody biomarker that targets IgG. It is present in 85% of patients with rheumatoid arthritis (RA) and about 5% of healthy controls (HC).

Spondyloarthropathy phenotype

Clinical presentation of the spondyloarthopathies demonstrates a number of shared phenotypic features. As their classification implies, they are linked by an inflammatory, axial arthritis. Patients also commonly present with peripheral arthritis, enthesitis, dactylitis, and a number of extra-articular manifestations that includes uveitis, psoriasis, and inflammatory bowel disease. Reports of cardiac, renal, and neurological issues are also noted. These core features are presented in Table 1.2. The spondyloarthropathies are also strongly associated with the class I major histocompatibility complex, HLA-B27. These associations, in Caucasians of western European

descent, are outlined in Table 1.3. Overall to diagnose SpA, the European Spondyloarthropathy Study Group criteria are commonly used, Table 1.4. These criteria capture a wide disease spectrum.

Ankylosing Spondylitis – Overview, genetic factors, and clinical diagnosis

AS is considered to be the hallmark spondyloarthropathy, and is also the most prevalent.[2] It is clinically defined by an insidious back pain in which the arthritis affects the spine and sacroiliac joints of the pelvis. Initially, this causes patients pain and stiffness, but as the disease progresses large numbers of patients experience ankylosis; the fusion of bones within the joint. Prior to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and biologics inhibiting TNF-α, patients would experience a striking deformity of the spine. Intriguingly, it is atypical for an autoimmune disease and is known to affect men more frequently than women at a ratio of 2:1 or even 3:1.[2] Additionally, the disease is particularly challenging because it presents relatively early, when a patient is in their 20's-30's.[4,5]

AS has a remarkable genetic association. Familial aggregation studies have estimated that genetic risk factors contribute to 80-90% of the disease.[6] Aligning with these estimates, 80-90% of patients with AS have the HLA-B27 gene. For 40 years HLA-B27 has been known to be a susceptibility gene[7], yet it's mechanistic contribution to the disease is still misunderstood. Animal models in HLA-B27, β2m transgenic rats develop spontaneous spondlyoarthritis-like disease.[8] Intriguingly, genetic ablation of the CD8 gene, which eliminates detectable CD8+ T cells, does not protect rats from spondyloarthritis only delaying the disease's presentation.[9] Adult thymectomy plus antibody mediated CD8 depletion also failed to protect HLA-B27 transgenic rats from arthritis and colitis.[10] However, these rats are protected from intestinal inflammation and peripheral joint disease when raised in a germfree environment.[11] This work suggests that commensal gut flora may contribute to pathogenesis. Such studies highlight the significance of HLA-B27, however it is

interesting to note that 10-20% of patients lack HLA-B27, but still develop disease and may represent a unique subset.

Beyond identifying the class I major histocompatibility complex HLA-B27, other genes known to play critical roles in adaptive immunity have been identified by GWAS as susceptibility genes for AS. Recently, a large cohort of 10,000+ AS patients and 15,000+ controls profiled by ImmunoChip (Illumina, San Diego, CA, USA) identified 29 immune-related risk loci.[12] 15 of these loci had been identified in an earlier study.[13] These results are summarized in Table 1.5.

The diagnosis of AS is predominantly made from clinical observations. In 1963, the diagnostic criteria for AS were specified at the Rome conference.[14] Later in 1966 it was updated, which led to the New York criteria for AS being used as the standard for diagnosis.[15] In 1984, to improve diagnostic specificity and sensitivity, van der Linden, Valkenburg, and Cats synthesized the two aforementioned clinical criteria to form the "modified New York criteria"; which remains the standard to this day.[16] The modified New York criteria are presented in Table 1.6. This diagnosis is centered on lower back pain, limitation of axial mobility, and a radiographic observation of sacroiliitis.

In addition to these cardinal features, AS patients also commonly present with a number of extra-articular features that include inflammatory eye involvement (acute anterior uveitis, iritis, and conjunctivitis), inflammatory bowel disease symptomology (colitis), skin involvement (psoriasis) and cardiac issues (aortic incompetence, aortitis, conduction disturbances, and heart block).[17–22]

Psoriatic arthritis – Overview, genetic factors, and clinical diagnosis

PsA is another spondyloarthropathy disease subset defined phenotypically by the scaly, red well-demarcated skin plaques of psoriasis (PsO) in conjunction with elements of axial and peripheral

arthritis. Patients may also present with enthesitis, dactylitis, and nail disease. In 1973, Moll and Wright defined PsA as a "psoriasis associated with inflammatory arthritis, and usually a negative serologic test for rheumatoid factor". Additionally, they proposed five subtypes: monoarthritis and oligoarthritis, polyarthritis, arthritis of distal interphalangeal joints with nail changes, arthritis mutilans, and spondylitis. Historically, there was a time period, when PsA was debated as a variant of RA, but it is now viewed as a distinct clinical entity.[23] Similar to the other spondyloarthropathies, extra-articular manifestations include uveitis and inflammatory bowel disease (IBD).[24] Comorbidities include obesity, dyslipidemia, type II diabetes, liver disease, and cardiovascular disease.[24–27]

The overall prevalence of PsA in the United States is estimated to be 0.25% (95% confidence interval [CI]: 0.18-0.31%). Typically, the presentation of psoriasis precedes arthritic features in PsA, and, in contrast to AS, the literature suggests that PsA demonstrates equal gender susceptibility.[28] HLA-B27 has been identified as a clinical predictor and is associated with a shorter time period between the onset of PsO and the onset of PsA.[29] IL-13 has also been demonstrated as a risk locus.[30] Additional susceptibility loci associated with antigen presentation, skin barrier function, innate immunity, and adaptive immunity were summarized by Sukhov and colleagues. These findings are presented in Table 1.7.[31]

PsA diagnosis is guided by the clinical observations outlined in the Classification Criteria for Psoriatic Arthritis (CASPAR). CASPAR relies on 5 key observations: evidence of psoriasis, nail dystrophy, dactylitis, seronegativity for RF, and radiological evidence of juxta-articular new bone formation. These criteria are outlined in Table 1.8. To be classified as having PsA a patient must have inflammatory articular disease affecting either the joint, spine, or enthesis with the presence of 3 or more of the 5 key CASPAR criteria. CASPAR has demonstrated high sensitivity and specificity.[32–34] Notably, nail involvement in the form of pitting, dystrophy, or oncholysis is a useful diagnostic clue

for clinicians. However, this diagnostic criteria is complicated by the observance that PsA is "usually" seronegative for RF. 10% of PsA patients, 10% of patients with uncomplicated psoriasis and 15% of the healthy population have RF present in their serum.[35,36] By comparison upwards of 80% of RA patients are RF positive.[37] Diagnosis is further complicated by the fact there is significant phenotypic heterogeneity between PsA patients.

Insights into the role of the adaptive immune system in AS and PsA

With regard to this inconsistent observation around RF, clinicians are limited by the lack of diagnostic biomarkers in both AS and PsA. Moreover, given the significant heterogeneity observed in the SpA and the overlapping phenotypic traits these diseases are commonly misdiagnosed and often diagnosis is delayed. Further, radiographic assessment only identifies a phase of disease that is fairly advanced in its course. This highlights the critical need for a robust biomarker. There is a strong association between SpA and HLA-B27, making HLA-B27 typing useful. However, it omits a significant SpA population that is HLA-B27 negative; a population that is nearly 20% of those afflicted with AS and 50% of PsA patients. Acute phase reactants have also been employed as a guide to diagnosis, but only 50% of AS patients had elevated C-reactive protein concentrations in one study.[38] Acute phase reactants were elevated in 40% of PsA patients. Additionally, an elevated erythrocyte sedimentation rate and leukocytosis were observed in one-third of PsA patients. Overall, laboratory assessments in the SpA lack specificity and reflect general inflammatory mediators.

In the autoimmune rheumatic diseases, the clinical utility of autoantibodies for biomarker use is well appreciated. Studies have shown that autoantibodies can be effective for early diagnosis, overall diagnosis, prognosis, response to treatment, and notably for identifying phenotypic subsets. For example, in rheumatoid arthritis (RA) autoantibodies targeting the enzyme PAD4 are associated

with more severe disease. In myositis (Myo), an autoimmune disorder targeting muscle, autoantibodies to aminoacyl tRNA synthetases are associated with the antisynthetase syndrome phenotype of myopathy. In this condition patients present with interstitial lung disease, nonerosive arthritis, and mechanic's hands. Autoantibodies targeting MDA-5 associate with dermatomyositis reflecting skin involvement and limited muscle damage. Similarly, in scleroderma autoantibodies targeting CENP A, B, and C associate with the limited phenotype restricted to patients extremities; and autoantibodies targeting topoisomerase I associate with the diffuse phenotype where skin thickening is observed broadly over the body. These findings were recently summarized in a review by Casciola-Rosen and Rosen, and are presented in Table 1.9.[39]

It remains contested whether SpA is driven by a targeted immune response from the adaptive immune system or whether it is an autoinflammatory condition driven by innate immune components. Due to this lack of clarity, it is also possible that another unknown mechanism exists. However, a number of insights do hint at a role for the adaptive immune system in AS and PsA pathology. This hypothesis is centered on the basis of 3 key findings:

1. immune cell infiltrates in the target tissue

2. reports identifying autoantibodies in both AS and PsA

3. the identification of numerous susceptibility alleles with well-defined functions in adaptive immunity (as discussed previously)

These hints suggest the possibility that a useful autoantibody biomarker may be present in these diseases.

It has been demonstrated via immunohistochemical studies in both AS and PsA that infiltrates are present at inflamed target tissue sites. Laloux and colleagues found CD3+, CD4+, CD8+,

and CD20+ infiltrates at sites of enthesis in all 7 HLA-B27 positive patients they studied in AS, as well as 1 HLA-B27 positive PsA patient. CD3+ infiltrates were elevated in SpA samples compared to enthesis infiltrates in RA, and CD8+ T cells were observed to be the dominant population.[40] Organized lymphoid aggregates resembling germinal centers have also been found ectopically in PsA synovial biopsies. In this study 13 out of 27 PsA synovial biopsies (48%) were observed to have large aggregates with organized T/B cell segregation, positive immunostaining for the lymph node homing chemokines CXCL13, CCL21, and CXCL12, and positive immunostaining for peripheral lymph node adressin (PNAd); a marker of the high endothelial venule (HEV).[41] Ectopic lymphoid follicles have also been observed in RA[42–45], where numerous autoantibodies have been identified.[37]

Further support for the role of the adaptive immune system in AS and PsA is provided by a number of recent reports identifying autoantibodies. In AS autoantibodies to Noggin, Sclerostin, CD74, PPM1A, and IL-6 have been reported. It is interesting to note that two of these autoantigens, CD74 and IL-6, have known immune functions. CD74 is the class II invariant chain, and IL-6 is an inflammatory cytokine. In PsA, autoreactivities to TWEAK, STIP1, N-Rap, progranulin, thyroid peroxidase, thyreoglobulin, and the 20s proteasome have been reported. Additionally, autoreactivity to the post-translational modifications carbamylation and citrullinated vimentin have also been observed. These results are summarized in Table 1.10.

In light of these connections between the adaptive immune system with AS and PsA, we hypothesize that a specific, antibody mediated response has a role in disease pathology. To assess this hypothesis we have interrogated patient serum in AS and PsA using a high throughput protein microarray consisting of 17,000 human proteins to broadly scan for the presence of autoantigens. Ultimately, the identification of a novel autoantibody has the potential for clinical use as a

diagnostic biomarker, and additionally may provide basic insights into the pathogenesis of these diseases.

CHAPTER II – THE IDENTIFICATION OF AUTOANTIBODIES TARGETING IFN-α AND TBX20 IN A COHORT OF PSORIATIC ARTHRITIS PATIENTS

Abstract

Objectives - The identification of autoantibodies in the autoimmune rheumatic diseases has proven critical in aiding clinical diagnosis, assessing disease activity, and for defining phenotypic subsets of patients. Here we outline a high throughput method of autoantibody identification using a human proteome array composed of 17,000 proteins. Using this serum profiling technique we were able to confirm the presence of 3 known and previously characterized myositis autoantibodies to HARS, MDA-5, and SNRP70. Additionally, we identified 3 other autoreactivities; to PUF60 in dermatomyositis, and IFN-α1 and TBX20 in a cohort of patients with psoriatic arthritis (PsA).

Methods – Autoantibodies were identified using a human proteome array. To assess the accuracy of the array technology, known myositis autoantibodies were used as positive controls. Validation of autoreactivities to IFN-α1 and TBX20, were identified in a cohort of PsA patient serum and assessed via immunoprecipitation assays combining patient serum with in vitro transcribed and translated (IVTT) radiolabeled recombinant protein. Serum was next screened by ELISA to better understand the frequency of these autoantibodies. For the ELISA, sera from patients with the following diseases were screened: psoriatic arthritis (PsA; n=58), ankylosying spondylitis (AS; n=20), rheumatoid arthritis (RA; n=30), and healthy controls (HC; n=26).

Results – The protein array identified 2 autoantibody specificities in our PsA cohort to IFN-α1 and TBX20. The results of our ELISA demonstrated IFN-α1 autoantibodies were present in 8.6% of PsA patients compared to a frequency of 0% in healthy controls. Additionally, TBX20 was identified as a novel autoantigen from our protein array screens. Follow-up ELISA experiments demonstrated that 13.8% of PsA patients had the presence of TBX20 autoantibodies, compared to 3.8% found in healthy controls.

Conclusions – Protein arrays can be useful tools to confirm, as well as identify novel autoantibody specificities.

INTRODUCTION

Psoriatic arthritis (PsA) is an inflammatory arthritis in which patients also commonly present with the characteristic red skin lesions of psoriasis.[46] It is most commonly defined by the presence of peripheral arthritis, axial arthritis, enthesitis, dactylitis, and skin and nail disease. Additionally, subsets of patients present with extraarticular manifestations that include ocular inflammation and inflammatory bowel disease [47]. PsA is predominantly diagnosed clinically using the international classification of psoriatic arthritis (CASPAR). To date a well-defined serum autoantibody associated with either diagnosis, prognosis, or disease activity is lacking. Consequently, PsA belongs to the family of seronegative spondyloarthopathies since the majority of patients do not have rheumatoid factor (RF) in their serum. [36,35]

The clinical utility of autoantibodies in the rheumatic diseases is well appreciated. In rheumatoid arthritis (RA) the presence of autoantibodies to the post-translational modification citrulline have proven critical for disease diagnosis, as well as being associated with a more aggressive course of disease.[48] In myositis, autoantibodies are useful to define clinical subgroups. For example, autoantibodies recognizing Mi-2, MDA5, TIF1γ, and NXP-2 preferentially associate with dermatomyositis. In contrast, autoantibodies to SRP and HMG-CoA reductase segregate with autoimmune necrotizing myopathies.[49] Additionally, within myositis, anti-synthetase autoantibodies are known to be indicative of a clinical subset that presents with interstitial lung disease.[50] Intriguingly, in the rheumatic disease systemic lupus erythematosus (SLE) 180 autoantibodies have been reported.[51]

PsA is hypothesized to have an autoimmune mechanism. In synovial biopsies from PsA patients the formation of ectopic lymphoid sites has been demonstrated. These sites show an organized follicle structure with T/B cell separation and are positive for high endothelial venule

(HEV) staining.[41] T cell infiltrates have also been observed in the seronegative spondyloarthropathy ankylosing spondylitis,[40] which shares a number of phenotypic characteristics with PsA. These findings would suggest the presence of organized, immune-mediated processes involving both the T and B cell arms of the adaptive immune response. However to date, serum analysis has revealed limited hints of a specific autoantibody response in PsA.

Recent reports of autoreactivities in PsA have suggested the presence of autoantibodies to native proteins, post-translational modifications, and pepide fragments. In their unmodified form TWEAK[52] and STIP1[53] have been reported as autoantigens in PsA. PsA patients have also been reported to have elevated levels of antibodies targeting cabamylated proteins[54] and mutated citrullinated vimentin.[55] Dolcino and colleagues have suggested that antibodies reactive to a PsA peptide crossreact with a number of proteins expressed in the skin and joint.[56] Additionally, antinuclear antibodies have been reported to be elevated in a PsA patient population.[57]

To gain a better understanding of these immune mediated processes we interrogated a protein microarray consisting of 17,000 human proteins with patient serum from a cohort of PsA patients.

METHODS

Patient cohorts

Patient serum samples for PsA was obtained from the Patient Centered Outcomes Research in Arthritis (PCORA, n=59). PsA diagnosis was confirmed in accordance with the CASPAR criteria. Patients with AS (n=20) consisted of samples provided by the National Institutes of Health. AS diagnosis was defined by the 1984 modified New York Criteria. Patients with rheumatoid arthritis (RA, n=30) were also obtained from the aforementioned PCORA study. Serum samples for patients recently diagnosed with Lyme disease (LD, n=20) came from the Study of Lyme Disease Immunology and Clinical Events (SLICE study) conducted at the Johns Hopkins Lyme Disease Clinical Research Center. In this study only untreated, early Lyme disease as defined by the CDC are enrolled. This LD cohort was only screened on the protein array. Healthy control sera (HC, n=26) also came from the SLICE study.

For protein array validation, myositis sera was used. Two reference serum samples were used, in which the reactivity was pooled from 4 total patients. Therefore, Reference 1 and Reference 2 each contained two autoantibody reactivities. The experimenter was blinded to these specificities and they were confirmed after autoantigen hit lists from the array were analyzed.

Serum profiling using human protein microarrays

Patient serum was profiled using a human protein array composed of >17,000 human proteins plus nonspecific controls. The HuProt arrays were obtained from CDI labs (Baltimore, MD). Each recombinant human protein contains an N-terminal GST-HisX6 tag and is spotted in duplicate on the array. Full length recombinant human proteins were purified from *Saccharomyces cerevisae*, as previously described.[1]

All steps in the development of the array were performed at room temperature. Arrays were first incubated for 1.5 hr with blocking buffer (3%BSA-TBST; 0.1% Tween-20, 1x Tris-buffered saline). Next, the arrays were probed with patient serum at a 1:5,000 dilution and rabbit anti-GST antibody diluted 1:10,000 from the manufacturer's stock (EMD Millipore; AB3282) in blocking buffer for a 1.5 hr incubation. Arrays were quickly washed with TBST and then underwent an extended wash of 5 minutes in TBST with shaking. This was repeated three times. After, the array was probed with the appropriate secondary antibodies diluted in blocking buffer for 1.5 h in the dark; donkey anti-human IgG (H+L) Cy5 [1:1,000] (Jackson ImmunoResearch, PA; 709-175-149) and goat anti-rabbit IgG Alexa 555 [1:1,000] (Invitrogen, CA; AB21428). Arrays were again washed as stated above, rinsed with double-distilled H_2O and dried by centrifugation.

Array Imaging and Analysis

Arrays were imaged using a GenePix 4000B microarray scanner (Molecular Devices, CA) and analyzed via GenePix Pro software. Signal intensity was Z-score transformed (Z_n) using the following formula $Z_n = (I_n - m)/\sigma$. In this equation, I_n equals the median foreground fluorescence intensity of the Cy5 channel at a single protein spot, m is equal to the median foreground fluorescence intensity of the Cy5 channel for all spots on the array, and σ is the standard deviation of m. Array hits in the disease populations were determined by subtracting out any hits in which a Z-score>5 was also found in healthy controls, and then sorting based off of patient frequency. The program Venny was used for Venn diagram analysis, kindly developed by J.C. Oliveros.

In vitro transcription and translation and immunoprecipitation (IVTT/IP)

Immunoprecipitation experiments were performed to validate protein array hits. IFN-α1 (NM_024013.1) and TBX20 (NM_020417.1) cDNA sequences were cloned into the pEF-Dest51

expression vector. 250 ng of cDNA was added to the Promega TnT T7 coupled reticulocyte lysate system with ^{35}S methionine to produce a radiolabeled, *in vitro* transcribed and translated (IVTT) protein of interest. The reaction was incubated for 1.5 hours at 30°C. After, 1-5 ul of this IVTT reaction was incubated with 1 ul of patient serum in 500 ul of NP40 lysis buffer (1% NP40, 20mM Tris, 150 mM NaCl, 1mM EDTA, pH 7.4) and immunoprecipitated with protein A agarose beads (Pierce, #20333). Beads were centrifuged, washed, and denatured with gel application buffer plus 2-mercaptoethanol. The immunoprecipitation product was electrophoresed on SDS-polyacrylamide gels and visualized by fluorography.

ELISA

A 96 well plate (Costar 9018) was coated overnight at 4°C with 50 ng/well of recombinant protein purchased from Origene; IFN-α1 (TP310902), TBX20 (TP328815). Washes were performed between this and all following steps at room temperature for 1 hour. Wells were blocked with 5% BSA in PBS/0.05% Tween-20 (PBST), then incubated with human serum diluted 1:500 in 1% BSA/PBST. Goat anti-human IgG conjugated to peroxidase was used as a secondary antibody (Jackson ImmunoResearch, 109-036-088). After washing, wells were incubated with SureBlue reagent (KPL) for a colorimetric readout at an absorbance of 450 nm. To allow for plate to plate normalization a positive reference serum sample was used at a dilution greater than 1:500. Samples were determined as autoantibody positive if they were ≥ 3 standard deviations (SD) above the mean of healthy controls. An ELISA data point was removed from the HC descriptive statistic results, since it was deemed an outlier via the Grubbs' test ($\alpha = 0.05$, cutoff).

RESULTS

Human proteome array screen confirms known autoantibodies to HARs, SNRP70, and MDA-5 in myositis serum

Prior to using the protein array to screen for the presence of potentially novel autoantibodies in PsA, we first assessed the accuracy of the technology by utilizing these arrays to interrogate sera from myositis patients with previously characterized and known autoantibodies. This was done in a blinded fashion using two reference sera as described in the Methods section. This approach was able to identify the correct autoantibody specificities to 3 well defined myositis autoantigens. Specifically, the autoantigen histidyl-transfer RNA synthetase (HisRS, Jo-1) was identified as the top hit with a Z-score of 27.1 (figure 2.1A) when Reference sera 1 was analyzed. An autoantibody response to HisRS is observed in up to 70% of patients with a myositis-interstitial lung disease (ILD) overlap syndrome[58–60], making it particularly useful in subsetting patients for disease phenotype. Also, correctly identified were the autoantigens MDA-5 and SNRP70 (figure 2.1B and 2.1C) which were found in Reference sera 2 and 1 respectively. The Z-score for MDA-5 was 18.4, and a Z-score of 9.7 was calculated for SNRP70. Of note, reactivities to these three proteins were not found in sera from patients with ankylosying spondylitis (AS), psoriatic arthritis (PsA), Lyme disease (LD), nor healthy controls (HC). Notably, all 3 previously characterized proteins were ranked in the top 11 of potential hits from our array lists. These results validated the effectiveness of the array technology in autoantigen identification, and thus gave us the confidence to utilize this approach in the identification of novel autoantigens.

The identification of a novel autoantibody response to PUF60

In collaboration with colleagues we were able to use the protein array to identify PUF60 as a novel autoantigen that is targeted in subsets of patients with Sjogren's syndrome (SS) and dermatomyositis (DM). The top PUF60 hit had a Z-score of 21.7 (figure 2.2A). Our array analysis also identified 2 isoforms of PUF60 in the top 15 of array hits (figure 2.2B). Autoantibodies to PUF60 were validated by proteomics, immunoblotting, and ELISA. This work observed PUF60 to be targeted in 30% of SS patients and 18% of DM patients.[61]

High throughput serum profiling of psoriatic arthritis identifies autoantibodies to IFN-α and TBX20

Having established the accuracy and specificity of the protein array technology, we next interrogated the arrays with sera from 19 PsA patients using 20 AS patients, 20 LD patients, and 18 healthy controls. The AS and LD sera were used as immune mediated disease specific controls. To threshold our data analysis we eliminated any hits in which a Z-score greater than 5 was also found in healthy controls. This analysis produced a list of 80 potential autoantigen targets that were specific to PsA (figure 2.3A). Within our top 20 hits (figure 2.3B), we noticed functional catagories of proteins (figure 2.3C) that displayed reactivities to tRNA synthetases, IFN-α isoforms, and proteins related to apoptosis. Intriguingly, when we compared the hit lists for each disease subset we observed minimal overlap between hits in AS, PsA, and LD (figure 2.4). These results suggested that possibly the protein arrays are able to identify disease specific signatures. The top 20 autoantigen hits in AS and LD can be viewed in Figure 2.5.

From our PsA specific hit list we followed up on IFN-α and TBX20 on the basis of their high Z-scores.

Validation of IFN-α as an autoantigen

Two isoforms of IFN-α were identified as targets on our hit list with robust Z-scores, reinforcing the strength of our analysis. The isoforms, IFNA1 and IFNA13, have a single amino acid difference present in the signal sequence of the protein. Thus, the mature secreted cytokines have identical protein sequences. In our initial array screen two serum samples displayed high Z-scores (figure 2.6A). To confirm the presence of autoantibodies to IFN-α in our patients we performed immunoprecipitation experiments using radiolabeled (^{35}S), in vitro transcribed and translated IFN-α1. Our IVTT/IP results confirmed the presence of autoantibodies against IFN-α in the 2 PsA patients initially identified (figure 2.6B). To gain a better understanding of the true frequency of this autoantibody in patients with PsA, we performed an ELISA to screen a larger number of patients, n=58. For the ELISA, positive samples were considered 3 standard deviations above the mean of healthy controls. Our results demonstrated that 8.6% of PsA patients (5/58) had high levels of autoantibodies to IFN- α, while no healthy controls were measured as positive (figure 2.6C). Additionally, we found particularly strong autoreactivity to IFN-α1 in one patientwith ankylosing spondylitis, N096 (figure 2.6D).

Further inspection of other IFN-α isoforms on the HuProt arrays demonstrated that PsA patients 213 and 135 also had elevated reactivity to the IFN-α14 and and IFN-α2 isoforms (figure 2.7). PsA patient 233 demonstrated elevated reactivity to IFN-α5 isoform (figure 2.7).

Validation of autoantibodies targeting TBX20

The T-box family member protein TBX20 was also identified as having a high a-score, 16.3, in one patient from our array screen (figure 2.8A). This protein is known to play a role in cardiac development[62], but has never been previously reported as an autoantigen. To validate this hit, we

once again followed-up with IVTT/IP experiments. The results of this experiment confirmed the presence of autoantibodies targeting TBX20 (figure 2.8B). To assess whether this autoantibody specificity was present in additional patients, we developed an ELISA and then screened a total of 58 serum samples from patients with PsA. Sera from other rheumatic diseases and healthy controls were also used. The ELISA results indicated that 13.8% of PsA patients have antibody reactivity to TBX20 (figure 2.8C).

DISCUSSION

In these studies we implemented a high throughput screen employing a protein microarray composed of 17,000 human proteins to identify the presence of autoantibodies in patients with PsA as well as other immune mediated inflammatory disorders. The arrays were successful in identifying well characterized myositis autoantibodies targeting HARs, SNRP70, and MDA-5. These proteins were all ranked within the top 11 on our list of potential hits. Additionally, this array technology led to the discovery of a novel autoantibody reactivity to PUF60. PUF60 autoantibodies were further validated by proteomics, immunoblotting, immunoprecipitation, and ELISA in this previously published study.[61]

Within our cohort of PsA patient serum we identified autoantibodies targeting the proteins IFN-α and Tbx20. These hits were identified via array and validated by IVTT/IP and ELISA. For IFN-α, the IFN-α1 and IFN-α13 isoforms, which have an identical mature protein sequence, were identified. Our ELISA demonstrated that 8.6% of PsA patients and 0% of healthy controls were autoantibody positive.

IFN-α is a secreted cytokine responsible for coordinating the host defense systems against intracellular pathogens. Primarily, it accomplishes this via the upregulation of antiviral, antiproliferative, and antigen processing proteins. IFN-α production is largely stimulated by viruses, but its role in anti-tumor responses has also garnered much interest. At the protein level there are 13 isoforms of IFN-α. Interestingly, our array screen identified strong autoreactivity to the isoforms IFN- α1 and IFN-α13. These isoforms have a single amino acid difference in the signal sequence, and thus the mature secreted proteins have identical sequences. This highlights the robustness of our analysis, since these proteins are printed at different locations on the array.

Previous reports have identified high titer autoantibodies to IFN-α2 in patients with autoimmune polyendocrinopathy syndrome type 1 (APS-1)[63] and myasthenia gravis and/or thymomas.[64] Recently, a protein array also identified seroreactivity to IFN-α1 and IFN-α13 in APS-1 patients.[65] APS-1 patients are deficient in the AIRE gene and in addition to autoantibodies, present with impaired endocrine function and chronic *Candida* infections.[66] It is interesting to note that IFN-α autoantibodies are known to be present in APS-1 patients with a deficiency in self-tolerance and are also elevated in the three autoimmune diseases we screened by ELISA. Further, it is interesting to note that we identified autoantibodies targeting an inflammatory cytokine; as it has been previously proposed that beneficial autoimmunity may work to dampen down this immune response. In this report investigators uncovered anti-TNF-α autoantibodies in a model of RA.[67] We must note that upon becoming unblinded to the patient's clinical work up, one IFN-α positive patient, 213, was identified as having myasthenia gravis and a benign thymoma. This may confound our result, as others have already identified IFN-α2 reactivity in patients with thymoma and/or myasthenia gravis.[64] We also note that one AS patient, N096, had a strong reactivity to IFN-α1; demonstrated by IVTT/IP.

We also identified autoantibodies targeting Tbx20, but more specifically the Tbx20b isoform. Our ELISA results demonstrated that Tbx20b autoreactivity was found in 13.8% of PsA patients. This is the first time Tbx20 has been reported as an autoantigen. There are 4 isoforms, of which Tbx20a and Tbx20b are the best studied. Tbx20b, isoform 2, is truncated at the C terminus in comparison to Tbx20a. Primarily, Tbx20 is known for its role in cardiac tissue development. In murine systems, it is reported to physically interact with NKX2-5, GATA4, and GATA5 to regulate gene programs involved in cardiac proliferation, chamber specification, and valvulogenesis.[68] Intriguingly, missense and nonsense mutations have been identified in human patients with congenital heart defects (CHD) and

adult cardiomyopathies.[62,69–71] In work by DeBenedittis and Jiao, it was observed that Tbx20a expression is restricted to the heart and Tbx20b expression has broader expression in the heart, body, and head of E12.5 mice lysates.[62] Largely, Tbx20 has been studied in human and murine systems, however it also plays an interesting role in the excretory pathway of zebrafish with an essential function in cloaca development. In this study, Tbx20 was found to be downstream of Bone Morphogenetic Protein (BMP) signaling.[72] In humans, BMP signaling plays a key role in bone and cartilage development[73], which is interesting to account for in our study of a rheumatic disease with observed bone erosions.

In future studies, it would be interesting to explore the possibility of a link between autoantibodies targeting Tbx20, a known regulator of cardiac tissue, and the cardiac abnormalities observed in subsets of PsA patients. Further, since Tbx20b is a shorter isoform it is also possible that these autoantibodies may be cross reactive to the Tbx20a isoform.

We also must note that we encountered some difficulty in validating a handful of HuProt Array hits in the AS, PsA, and LD serum samples that were screened by this approach. It is possible that our primary validation experiments of IVTT/IP and ELISA may have hidden epitopes that were available for antibody binding on the protein arrays. In IVTT/IP and ELISA experiments we assume the target protein is in its tertiary structure. However, since the HuProt arrays are coated in nitrocellulose it is possible that the proteins printed on the array are denatured and thus largely linear epitopes are available for antibody binding. Consequently, these linear epitopes could have been masked, as a result of protein folding, in our IVTT/IP and ELISA validation experiments. It is also possible that the HuProt array screens are a more sensitive approach in comparison to IVTT/IP and ELISA. If true, validation would be difficult since current and accepted validation techniques are not as sensitive as our array screens.

Overall, we believe the use of human proteome arrays to screen for the presence of autoantigens represents a promising technique to uncover high-titer autoantibodies in the autoimmune rheumatic diseases. Here we have identified the immune cytokine IFN-α1 and the cardiac transcription factor Tbx20 as autoantigens. Further studies may help to elucidate whether these autoantibodies can be utilized in disease diagnosis, correlated with disease activity, or for subsetting of phenotypically distinct patients.

TABLES

Table 1.1 – Criteria for members of the seronegative spondarthritis

Criteria for members of the seronegative spondarthritis
1. Negative tests for rheumatoid factor 2. Absence of subcutaneous (“rheumatoid”) nodules 3. Inflammatory peripheral arthritis 4. Radiological sacroiliitis with or without classical ankylosying spondylitis 5. Evidence of clinical `overlap’ between members of the group A. 2 or more of the following features: psoriasiform skin or nail lesions; ocular inflammation including conjunctivitis or anterior uveitis; buccal ulceration; ulceration of the small or large intestine; genital ulceration; genito-urinary infection (particularly urethritis and/or prostatitis; erythema nodosum; pyoderma gangrenosum; thrombophlebitis). 6. Tendency of familial aggregation
Source: Moll and Wright 1974

Table 1.2 - General features of the spondyloarthropathies

• Radiographic sacroiliitis with or without accompanying spondylitis • Variable inflammatory peripheral arthritis, enthesitis, and dactylitis • Association with chronic inflammatory bowel disease • Association with psoriasis and other mucocutaneous lesions • Tendency for anterior ocular inflammation • Increased familial incidence • Occasional aortitis and heart block • No association with rheumatoid factor (RF) • Strong association with HLA-B27
Source: Khan, M.A. *Ann. Intern. Med. (2002)*

Table 1.3 – Spondyloarthropathy association with HLA-B27 in caucasians of western European descent

Disease	HLA-B27 prevalence (approximate)
Ankylosing spondylitis	80-90%
Reactive arthritis	40-80%
Juvenille spondyloarthropathy	70%
Enteropathic spondyloarthritis	35-75%
Psoriatic spondyloarthritis	40-50%
Undifferntiated spondyloarthropathy	70%
Acute anterior uveitis (acute iritis)	50%
Aortic incompetence with heart block	80%

Source: Khan, M.A. *Ann. Intern. Med. (2002)*

Table 1.4 – The European Spondyloarthropathy Study Group Criteria

Criteria	Definition
A. Inflammatory spinal pain; with ≥ 4 of the following 5 components 1. At least 3 months in duration 2. Onset before 45 years of age 3. Insidious (gradual) onset 4. Improved by exercise 5. Associated with morning spinal stiffness	History of or current symptoms of spinal pain (low, middle, and upper back or neck region)
B. Synovitis	Past or present asymmetric arhritis, or arthritis in the lower limbs
C. Spondyloarthropathy	Presence of inflammatory spinal pain OR synovitis AND one or more of the following conditions: - Family history: first or second degree relatives with ankylosing spondylitis, psoriasis, acute iritis, reactive arthritis, or inflammatory bowel disease - Past or present psoriasis - Past or present ulcerative colitis or Crohn's disease - Past or present pain alternating between the two buttocks - Past or present enthesitis - Episode of diarrhea occuring 1 month before the onset of arthritis - Nongonococcal urethritis or cervicitis occuring 1 month before the onset of arthritis - Bilateral grade 2-4 sacroiliitis or unilateral grade 3-4 sacroiliitis

Source: Khan, M.A. *Ann. Intern. Med. (2002)*

Table 1.5 – Ankylosing spondylitis susceptibility genes/loci identified by genome-wide association studies

Summary of ankylosing spondylitis susceptibility genes/loci identified by genome-wide association studies	
Loci previously associated with AS	
RUNX3	Runt-related transcription factor 3
IL23R	Interleukin 23 receptor
GPR25	G-protein-coupled receptor 25
KIF21B	Kinesin family member 21B
Intergenic	Chromosome 2p15
PTGER4	Prostaglandin E receptor 4 (subtype EP_4)
ERAP1	Endoplasmic reticulum aminopeptidase 1
IL12B	Interleukin 12B
CARD9	Caspase recruitment-domain family member 9
LTBR	Lymphotoxin β-receptor (TNFR superfamily, member 3
TNFRSF1A	Tumor-necrosis factor-receptor superfamily member 1A
NPEPPS	Aminopeptidase puromycin-sensitive
TBKBP1	TNFR-associated factor family member-associated nuclear factor-κβ-binding kinase I-binding protein
TBX21	T-box 21
Intergenic	Chromosome 21q22
New loci associated with AS	
IL6R	Interleukin 6 receptor
FCGR2A	Fc fragment of immunoglobulin G, low-affinity IIa, receptor (CD32)
UBE2E3	Ubiquitin-conjugating enzyme E2E 3
GPR35	G-protein-coupled receptor 35
BACH2	BTB and CNC homology 1, basic leucine-zipper transcription-factor 2
ZMIZ1	Zinc finger, MIZ type-containing 1
NKX2-3	NK2 homeobox 3
SH2B3	Src homology 2B adaptor protein 3
GPR65	G-protein-coupled receptor 65
IL27	Interleukin 27
SULT1A1	Sulfotransferase family cytosolic 1A
NOS2	Nitric oxide synthase 2
TYK2	Tyrosine kinase 2
ICOSLG	Inducible T-cell costimulator ligand

Table 1.6 – The modified New York Criteria for ankylosing spondylitis diagnosis

Modified New York Criteria for Ankylosing Spondylitis (1984)
Diagnosis Clinical criteria 1. Low back pain for at least 3 months, improved by exercise and not relieved by rest. 2. Limitation of lumbar spine motion in saggittal and frontal planes. 3. Chest expansion decreased relative to normal values for age and sex Radiographic criteria 4a. Unilateral sacroiliitis grade 3-4 4b. Bilateral sacroiliitis grade 2-4
Grading 1. Definite AS if (4a or 4b) AND any clinical criterion (1-3) 2. Probable AS if: a. 3 clinical criteria are present b. The radiographic criterion is present without any signs or symptoms satisfying the clinical criteria. (Other causes of sacroiliitis should be considered)
Source: Van der Linden et al., *Arthritis Rheum* 1984; 27: 361-368

Table 1.7 – Psoriatic arthritis susceptibility genes/loci

Antigen presentation	
*HLA-Cw*0602*	Strong association with PsV, earlier disease onset and positive family history
HLA-B27	Risk allele for axial joint disease in PsA
HLA-B39	Associated with PsA; similar to HLA-B27 encodes electronegative B pockets
HLA-B40	Protective against PsA; encodes electropositive B pockets
ERAP1	Variants have decreased aminopeptidase activity involved in antigen processing
PSMA6	Encodes a subunit involved in MHC class I antigen processing
Skin barrier function	
LCE3	*LCE3C_LCEB-del* is thought to increase susceptibility to exogenous substances due to impaired barrier function in PsV
Innate immunity	
CARD14	Activates NF-κβ which upregulates IL-8 AND CCL20 in skin, and controls early osteoclast differentiation in bone
NOS2	Encodes inducible nitric oxide synthase, which has increased expression in psoriatic plaques and synovium
TNIP1, TNFASIP3	These genes control the ubiquitination and degradation of Iκβ-a, which inhibits NF-κβ signaling by retaining it in the cytoplasm
NFKBIA	Encodes Iκβ-a
Adaptive immunity	
IL12B	Increased IL12B gene expression has been found in psoriatic plaques
IL23R, IL23A	Th17 associated phenotype
IL12RB2, TYK2	IL-12 receptor plays a key role in the Th1 phenotype. *TYK2* is required for IL-12B1 signaling
TRAF3IP2	Encodes the adaptor protein CIKS (Act1), which has a role in IL-17 mediated inflammation
Source: Sukhov, A. *Clin. Rev. Allergy Immunol.* (2016)	

Table 1.8 – The CASPAR classification criteria for PsA

Grading: To be classified as having PsA, a patient must have inflammatory articular disease (joint, spine, enthesis) with ≥ 3 of the following 5 points:	
Criterion	**Description**
1. Evidence of psoriasis (one of a, b, or c)	
a. Current psoriasis[a]	Psoriatic skin or scalp disease currently present, as judged by a rheumatologist or dermatologist
b. Personal history of psoriasis	A history of psoriasis obtained from a patient or family physician, dermatologist, rheumatologist, or other qualified healthcare professional
c. Family history of psoriasis	A history of psoriasis in a first- or second-degree relative by patient report
2. Psoriatic nail dystrophy	Typical psoriatic nail dystrophy, including onchyolysis, pitting, and hyperkeratosis observed on current physical examination
3. Negative test for RF	By any method except latex, but preferably ELISA or nephelometry, according to the local laboratory reference range
4. Dactylitis	
a. Current	Swelling of an entire digit
b. History	A history of dactylitis recorded by a rheumatologist
5. Radiological evidence of juxta-articular new bone formation	Ill-defined ossification near joint margins (excluding osteophyte formation) on plain x-ray films of hand or foot

CASPAR, ClASiffication criteria for Psoriatic Arthritis; RF. rheumatoid factor; ELISA, enzyme-linked immunosorbent assay

[a] Current psoriasis scores 2; all other items score 1

Source: Primer on Rhuematic Diseases

Table 1.9 – Examples of phenotype and autoantibody association

Autoantigen	Phenotypic features
Autoimmune myopathies	
Aminoacyl tRNA synthetases	Antisynthetase syndrome: myopathy, interstitial lung disease, nonerosive arthritis, fever, and mechanic's hands
MDA5	Dermatomyositis with mild or absent muscle disease
Scleroderma	
Topoisomerase I	Diffuse cutaneous scleroderma
CENP A,B, and C	Limited cutaneous scleroderma
Rheumatoid Arthritis	
PAD4	More severe disease
Source: Rosen, A. *Annu. Rev. Immunol* (2016)	

Table 1.10 – Reported autoantigens in AS and PsA

Autoantigens in AS	
Autoantigen	Author
Noggin	Tsui, F. *Ann. Rheum Dis.* (2014)
Sclerostin	Tsui, F. *Ann. Rheum Dis.* (2014)
CD74	Baerlecken, NT. *Ann. Rheum Dis.* (2014)
PPM1A	Kim, YG. *Arthritis Rheumatol.* (2014)
IL-6	Wright, C. *Mol. Cell Proteomics* (2012)
Autoantigens in PsA	
Tweak1	Guis, S. *J. Transl. Med.* (2016)
STIP1	Maejima, H. *PLOS ONE* (2014)
N-Rap	Dolcino, M. *PLOS ONE* (2014)
Progranulin	Thurner, L. *Arthritis Res. Ther.* (2013)
Thyroid peroxidase	Peluso, R. *J Rheumatol* (2011)
Thyreoglobulin	Peluso, R. *J Rheumatol* (2011)
20s proteasome	Colmenga, I. . *J Rheumatol* (2008)
Carbamylation	Chimenti, M.S. *Autoimmun. Rev.* (2015)
Citrullinated vimentin	Dalmady, S. *Clin. Dev. Immunol.* (2013)

FIGURES

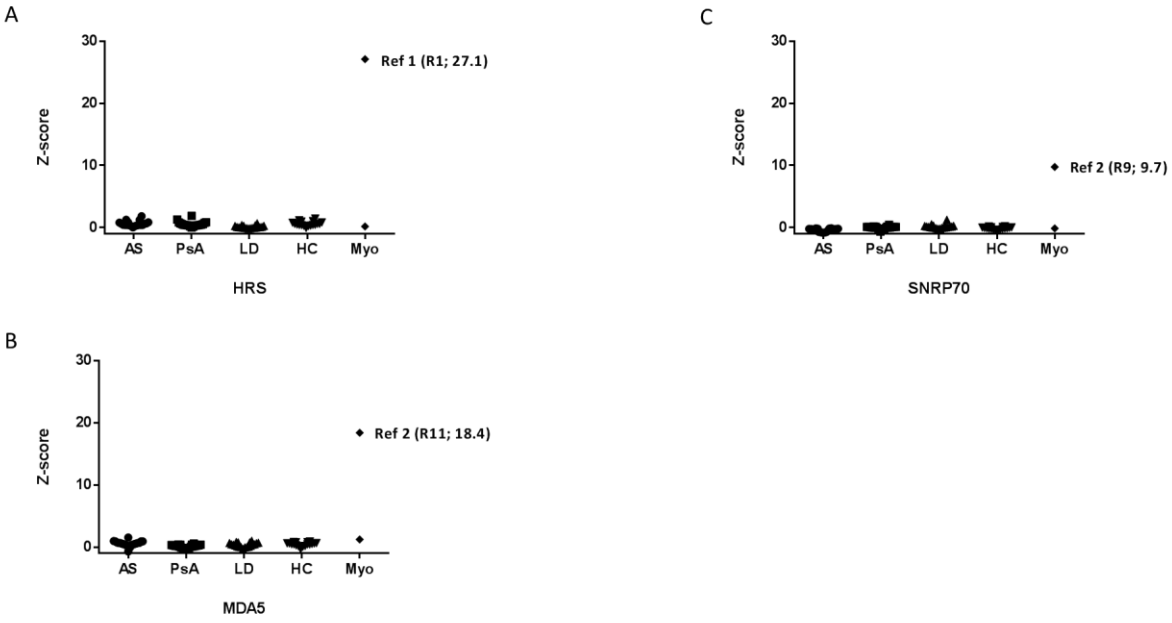


Figure 2.1 The HuProt array accurately identifies the presence of 3 autoantibodies in myositis sera. Patient serum at a 1:5,000 dilution was incubated with the HuProt for 1 hour. Serum samples included ankylosing spondylitis (AS, n=20), psoriatic arthritis (PsA, n=19), Lyme disease (LD, n=20) and myositis (Myo, n=2). (A) Histidyl-tRNA synthetase (HARS, Jo-1) was the top ranked protein (R1) identified in myositis sera (B) The known autoantigen MDA-5 was indentified as the 11th (R11) ranked protein in myositis sera. (C) SNRP70 is identified as an autoantigen and was the 9th ranked protein (R9) on the array.

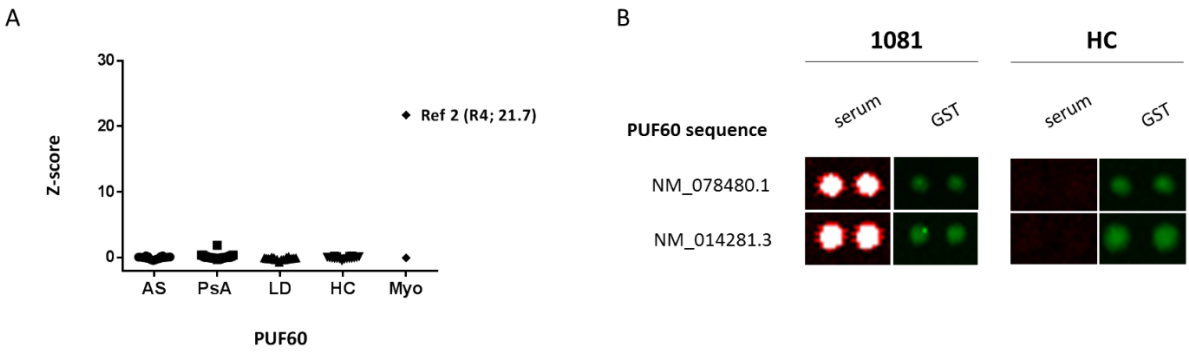
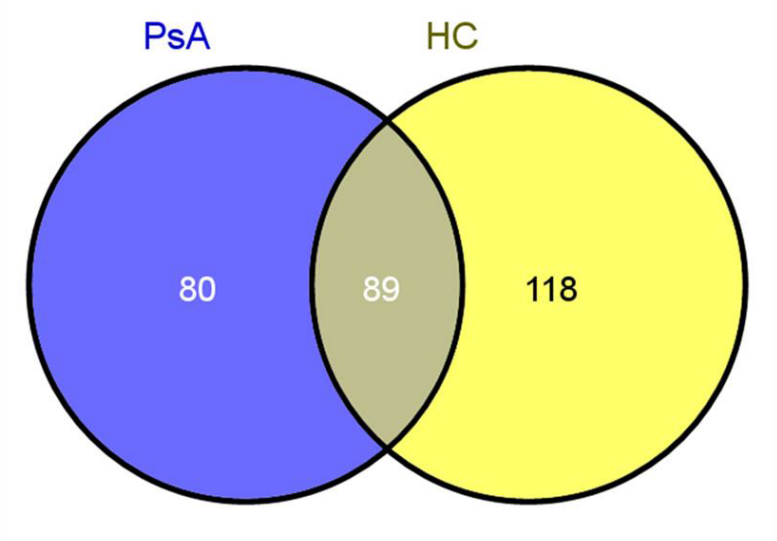


Figure 2.2 The identification of PUF60, a novel autoantigen, in a patient with dermatomyositis. (A) The HuProt array was incubated with dermatomyositis patient serum. An isoform of PUF60 was identified as the 4th ranked protein on our hit list, with a Z-score of 21.7. (B) Image analysis of the protein array demonstrated that autoantibodies in patient serum reacted to multiple isoforms of PUF60. Reactivity was not observed in healthy control samples

A



B

Top 20 array hits in PsA

Protein	Z-score ≥ 3
GlyRS – GARS	5
IFI6	4
TrpRS – WARS	3
SIRT1	2
NAPB	2
APOPT1	2
CCDC102B	2
IFNA1	2
TBX20	2
OR10AG1	2
IFNA13	2
HEMGN	2
TMEM211	2
AspRS – DARS	2
DNAJC12	2
KIAA1026	2
SPATA1	1
MAGEB10	1
ATP6V0C	1
MOCS2	1

C

Themes from hit list

tRNA synthetase	IFN-α	Apoptosis	Testicular expression
GlyRS	IFNA1	IFI6	SPATA1
TrpRS	IFNA13	APOPT1	MAGEB10
AspRS			

Figure 2.3 High throughput data analysis identifies 80 potential autoantigens specific to PsA (A) Venn diagram analysis demonstrates that at a cut-off of Z-score ≥ 5, there are 80 potential autoantigens found in PsA patients, but not healthy controls (HC). (B) A list of the Top 20 hits sorted on the frequency of PsA patients with Z-scores ≥ 3. (C) Functional themes identified in the Top 20 hit list.

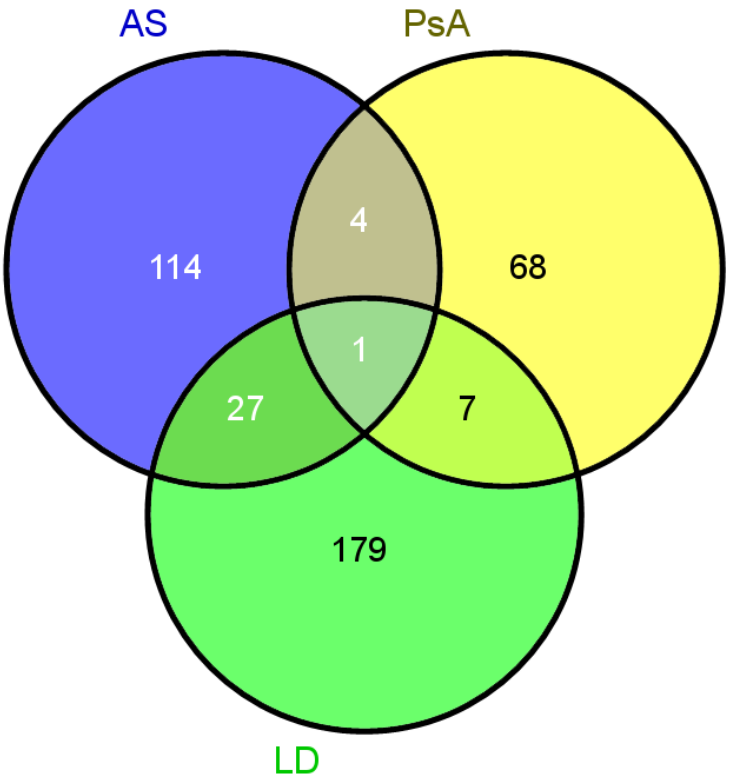


Figure 2.4 The HuProt array identifies unique autoantigen signatures present in AS, PsA, and LD. Hit lists specific to each disease subset were analyzed via Venn diagram demonstrating minimal overlap between the autoantigens recognized in each disease.

A

Top 20 array hits in AS

Protein	Z-score ≥ 3
C20orf19	3
MAST4	3
C1orf96	2
SNRK	2
KIAA0515	2
CABP4	2
RABEP2	2
RUNX1T1	2
APEG1	2
XRCC1	2
EEF2K	2
BCL3	1
RWDD1	1
MYL5	1
TPT1	1
RUNX1T1	1
RGS3	1
CAPS	1
TERF2IP	1
ASAP	1

B

Top 20 array hits in LD

Protein	Z-score ≥ 3
IGHG1	11
IGHV4	10
C12orf61	8
RAD23B	5
CNN2	4
ZRANB2	4
PPM1F	3
HS1BP3	3
LILRB5	3
SAP30L	3
SCG2	3
GAP43	3
ZADH1	3
BCL2L11	3
GPBP1	3
RBM12	3
LSP1	2
COL4A3BP	2
PTMS	2
KIAA1598	2

Figure 2.5 The Top 20 autoantigen hits identified in AS and LD. Lists are sorted based off a frequency of patient serum samples with Z-scores ≥ 3.

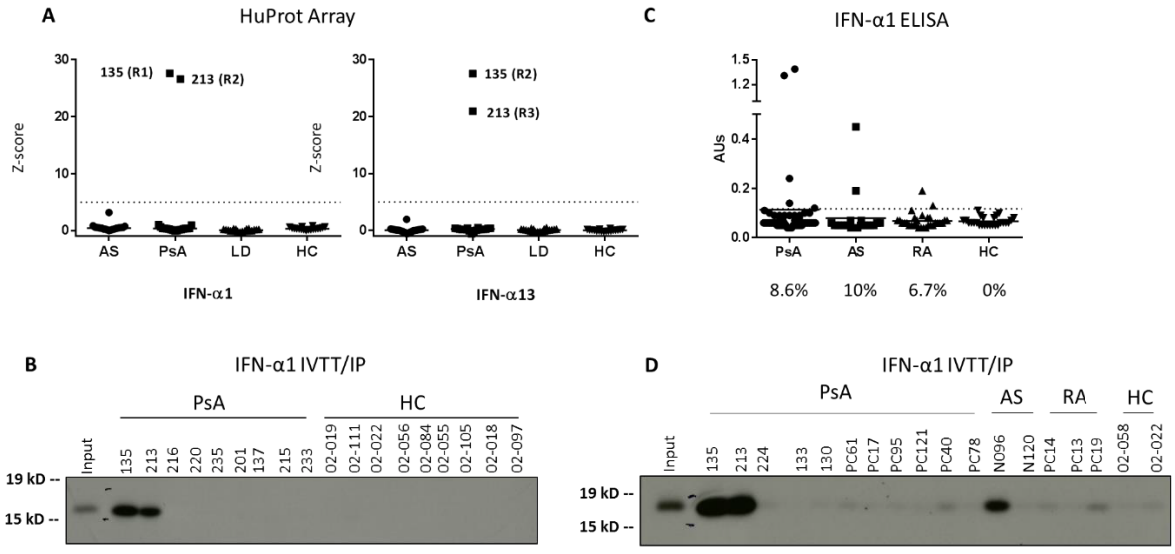


Figure 2.6 Autoantibodies targeting IFN-α are present in a cohort of PsA patients. (A) Protein array results demonstrate that the initial screen identifies 2 patients reacting to the IFN-α1 and IFN-α13 isoforms. Z-score ≥ 5 represented by the dotted line. (B) Radiolabeled IFN-α1 is immunoprecipated by patient sera. (C) An IFN-α1 coated ELISA using patient serum at 1:500. Percent positive is indicated. Dotted line represents the cut-off for positive antibody status as described in the methods. (D) IFN-α1 is also immunoprecipitated by an AS patient (N096).

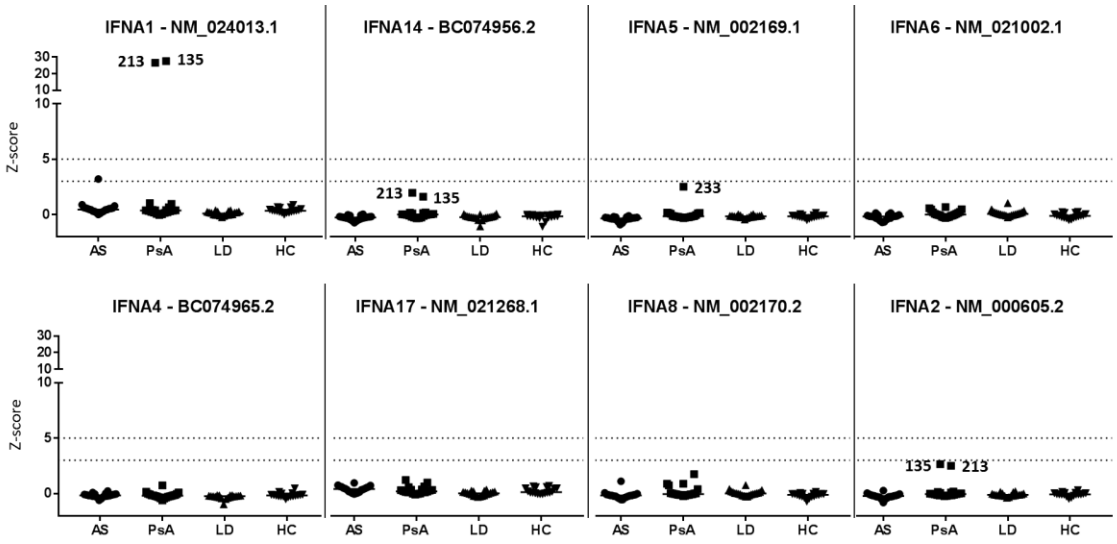


Figure 2.7 IFN-α isoform autoreactivities measured by the HuProt array. PsA patients 213 and 135 demonstrate elevated autoreactivity to IFN-α1, IFN-α14, and IFN-α2. PsA patient 233 has elevated autoreactivity to IFN-α5. Dotted lines represent Z-scores ≥ 3 and ≥ 5.

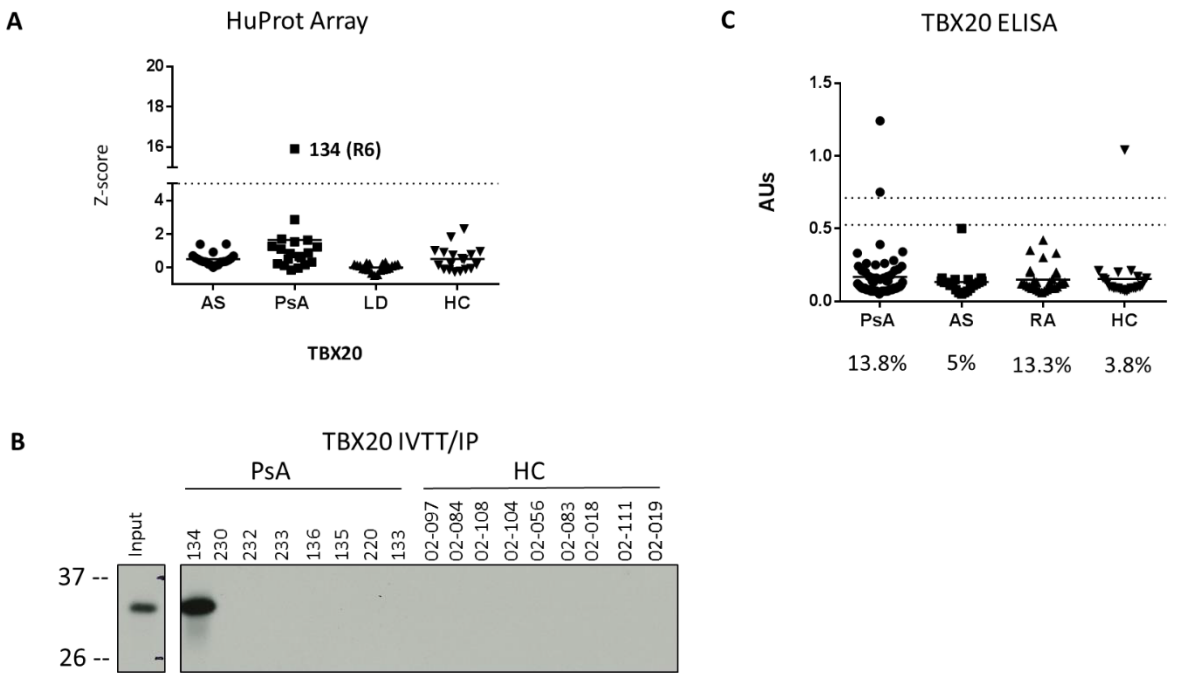


Figure 2.8 The identification of autoantibodies targeting TBX20. (A) TBX20 is identified as a hit in patient 134 with PsA, Z-score = 15.9. Z-score ≥ 5 represented by the dotted line. (B) IVTT/IP experiments confirm the presence of antibodies recognizing TBX20 in patient 134. (C) Antibodies against TBX20 were assayed by ELISA; patient serum at 1:500. The dotted line represents the cut-off for positive antibody status as described in the methods. Antibodies were detected in 13.8% of PsA patients, 5% of AS patients, 13.3% of RA patients, and 3.8% of HC.

CHAPTER III – INTRODUCTION: NONCLASSICAL MHC Ib IN T CELL DEVELOPMENT

INTRODUCTION

CD8+ effector function

CD8+ T cells play a critical role in the body's response to intracellular bacteria, viruses, and tumors. By recognizing foreign or mutated peptides presented by the major histocompatibility complex (MHC) class I, they are capable of eliminating infected, oncogenic, and even stressed cells. This process is known as cell-mediated immunity, and is critical for limiting and stopping the damage caused by disease.

In the periphery, this effector function, is composed of five key steps. First, the T cell receptor (TCR) of the CD8+ T cell recognizes peptide in the context of MHC class I on a target cell. Upon antigen recognition, TCRs will aggregate and dimerize at the central supramolecular activation complex (c-SMAC). This TCR clustering results in an intracellular signaling cascade initiated from the CD3 co-receptor complex and leading to the phosphorylation of intracellular activation motifs (ITAMs). The downstream components of this signaling result in the polarized release of granules containing three CD8 effector molecules; perforin, granzyme, and granulysin. These molecules are released at the immunological synapse and exquisitely directed at the target cell. This directed release allows the serine protease granzyme to enter the cytosol of the target cell via complexes of perforin and sergylcin. Here, granzyme activates a caspase cascade that culminates in target cell apoptosis; thus ridding the body of an infected or transformed cell.

In summary the five key steps described are: 1) Epitope recognition by the TCR 2) Immune synapse formation 3) Intracellular signaling 4) Granule release 5) Target cell apoptosis.

CD8+ T cell development

However, before a CD8+ T cell can play this vital role of immune surveillance, it must first undergo an intricate and rigorous developmental process. The core of this developmental process resides in the thymus. Within this setting, a developing T cell must refine the ability to recognize self-MHC while also balancing the task of avoiding auto-reactivity. This stage of development has been termed “T cell selection”, and the dilemma faced parallels that of the fairy tale protagonist Goldilocks. Ultimately, the T cell must develop its antigen receptor to be “just right”. To develop an appropriate TCR repertoire thymocytes, the T cell precursors, undergo positive and negative selection as they migrate through the cortex and medulla regions of the thymus.

With regard to T cells that eventually become CD8+ their TCR must recognize MHC class I during development. The ability of an immature thymocyte to recognize self-peptide:MHC class I complexes with intermediate affinity allows it to be positively selected. This recognition leads to a signal that up-regulates TCR expression and ceases expression of the CD4 co-receptor. At this time, CD8 expression is maintained on the cell surface. Now, as a single positive thymocyte, the CD8+ T cell continues to migrate through the thymus needing to avoid negative selection if it is to survive and mature into the periphery. It is understood that during negative selection auto-reactive cells are eliminated. Negative selection is initiated by strong recognition of self-peptide:MHC and leads to thymocyte apoptosis.

Studies on T cell development and TCR restriction have primarily focused on the role of the highly polymorphic, classical MHC class Ia molecules (MHC Ia). However, only recently has the focus shifted to the role being played by non-classical MHC class Ib (MHC Ib) molecules during T cell development. At the genetic level, non-classical MHC Ib molecules are defined by their incredibly low polymorphism. This level of evolutionary conservation stands in stark contrast to the hundreds

of alleles identified for class Ia molecules. Further, it has been demonstrated that classical MHC Ia selection occurs on thymic epithelial cells (TECs), while non-classical MHC Ib selection can be orchestrated by both TECs and hematopoietic cells (HCs).[74,75] Intriguingly, studies have shown that MHC Ib-restricted (nonclassical) CD8+ T cells have a distinct "innate-like" phenotype, in comparison to MHC Ia-restricted (classical) CD8+ T cells. This innate-like phenotype is defined by high levels of CD44 expression on naïve, murine T cells[76], and the ability to rapidly produce cytokines upon in vitro stimulation. Additionally, MHC Ib restricted T cells have been observed to mount a quicker immune response to *Listeria monocytogenes* (LM).[76,77]

Qa-1 overview

Qa-1 is an MHC Ib molecule with only four known alleles.[78] Predominantly, it displays a single peptide within its' hydrophobic binding pocket. This peptide is derived from the proteasome processed signal sequence of the MHC Ia molecules H-2D and H-2L; and requires TAP (transporter associated with antigen processing) dependent transport into the endoplasmic reticulum (ER). This peptide has been named the Qa-1 determinant modifier or Qdm. Qdm is composed of the nonameric amino acid sequence AMAPRTLLL. Peptide elution studies have demonstrated that Qdm comprises 70% of the total peptide content of Qa-1.[79]

Moreover, it is important to note that Qa-1 is the otholog to the human HLA-E molecule. Despite only 73% amino acid sequence homology, HLA-E largely presents a similar Qdm peptide with the sequence VMAPRTLLL. HLA-E is also non-polymorphic having only two known alleles in the human population.[78] Functionally, these orthologs have similar roles in the murine and human immune system, highlighting the translational appeal of studying Qa-1.

Qa-1 in missing self recognition

Qa-1 is most well studied for its' role as a natural killer (NK) cell receptor. The Qa-1:Qdm complex serves as a ligand for particular NKG2 family receptors that form heterodimers with CD94. A higher binding affinity has been demonstrated for the inhibitory CD94/NKG2A receptor, in comparison to the activating CD94/NKG2C/E receptors.[80] Consequently, this role positions Qa-1 at a critical node of antigen processing surveillance. Under normal conditions appropriate levels of Qa-1:Qdm on the cell surface transmits an inhibitory signal to probing NK cells and protects the cell from lysis. However, during times of MHC Ia downregulation, which may result from viral infection or tumorigenesis, lowered availability of Qdm reduces surface levels of Qa-1:Qdm, thus making these cells more susceptible to NK cell mediated lysis in a process that has been termed "missing self" recognition.[81] This interaction also crosses over into adaptive immunity, where it has been observed that approximately 5% of circulating CD8+ T cells are double positive for CD94 and NKG2A expression.[82]

Diverse peptide presentation by Qa-1

Despite the dominance of Qdm presentation by Qa-1, multiple groups have reported its' ability to present a diverse array of foreign and self-peptides. During intracellular bacterial infection Qa-1 can present epitopes from *Salmonella typhimurium*,[83] and Qa-1 restricted αβ CTLs have been identified in mice after infection with LM.[84–88] A greater number of pathogen derived peptides have been found to bind HLA-E that include: *Salmonella*, *Listeria monocytogenes*, LCMV, HIV, EBV, HCV, and *Mycobacterium tuberculosis*.[89,90] Numerous self-peptides have also been identified that bind Qa-1 including HSP60 in the absence of QDM[91], Fam49b in ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing) knockout cells[92], and an additional eighty-four

in TAP deficient cell lines.[93] These observations provide insights into Qa-1's ability to monitor antigen processing defects in an adaptive immune context, as a ligand for the TCR of CD8+ T cells.

CD8+ regulatory T cells

Additionally, there are a number of reports suggesting that Qa-1 restricted αβ T cells may play an important regulatory role. The observance that injecting naïve rats with an irradiated (or mitomycin C treated), myelin basic protein specific, CD4+ T cell line protects these rats from the induction of experimental allergic encephalomyelitis (EAE) has opened the door for further inquiry. This therapeutic strategy has been termed T cell vaccination (TCV). Studies of TCV in humans and rats have identified a CD8+ T cell population that specifically recognizes the pathogenic, vaccine T cells.[94,95] Work from the laboratory of Leonard Chess's lab has identified Vβ8-specific Qa-1-restricted CD8+ T cells that are induced by TCV and capable of lysing a CD4+ T cell clone that expresses Vβ8.[96] Recently, new observations have suggested that Qa-1 restricted CD8+ regulatory cells (CD8+ Tregs) monitor follicular T helper cells (Tfh). Using a genetic model to disrupt TCR/CD8 recognition of Qa-1, work by Kim and colleagues uncovered the development of a systemic-lupus-erythematosus-like autoimmune disease driven by autoantibodies.[97] Moreover, in a mouse model of collagen-induced arthritis these CD8+ Tregs were observed to eliminate autoreactive Tfh and Th17 cells, thereby inhibiting disease development.[98]

The observations that Qa-1 can alert CD8+ T cells of the adaptive immune system to antigen processing defects, in combination with research bridging an innate phenotype associated with positive selection by MHC Ib on HCs hints at an intriguing role for Qa-1. Is it possible that these cells have evolved to rapidly eliminate dangerous cells with compromised antigen processing systems? Such a role may prove significant since viral infection, stress, and tumorigenesis have all been demonstrated to impede appropriate antigen processing. In these circumstances the role of Qa-1

and HLA-E is understudied. Further, little clarity has been provided on the role CD8+ Qa-1-restricted T cells have in a regulatory context. Here we outline the development of a transgenic mouse model to investigate these hypotheses. This system will allow us to study the role played by Qa-1 in nonclassical T cell development and determine if positive selection can impart an innate phenotype on CD8+ T cells. Additionally, we aim to assess whether the existence of an innate, positively selected Qa-1 CD8+ T cell population has evolved to provide immunosurveillance against antigen processing defects. Further, it is possible that this reductionist system will lead to an enrichment of the CD8+ Treg population, and thus make this rare cell lineage easier to study. During the development of this mouse model, we have also optimized a flow-cytometry based CTL killing assay to serve as a replacement for the dated and cumbersome ^{51}Cr release assay. This method is also described.

CHAPTER IV – THE DEVELOPMENT OF A scQa-1^{b} MOUSE MODEL TO ASSESS T CELL DEVELOPMENT AND PHENOTYPE

ABSTRACT

Objectives – We have designed 3 DNA constructs that covalently link the Qa-1^b sequence to β_2-microglobulin (β2m) with the overall goal of selecting the construct that best mimics native Qa-1^b function for use in a transgenic mouse model. We refer to these constructs as single-chain Qa-1^b (scQa-1^b). Upon breeding this scQa-1^b transgenic with mice deficient in β2m -/-, we will have designed a system where Qa-1^b is the sole MHC I molecule present for T cell selection. This strategy will provide a unique, reductionist environment to study nonclassical, Qa-1 dependent T cell development and further interrogate CD8+ T cells present in the periphery. Peripheral CD8+ T cells will be thoroughly investigated with regard to localization, phenotype, TCR repertoire, cytokine secretion, and proliferation abilities.

Methods – 3 DNA constructs were cloned for potential transgenic use. These transgenes (Tg) contained either the genomic DNA (gDNA) or complementary DNA (cDNA) sequence of Qa-1^b followed by a repeating linker sequence to connect it to the sequence encoding β2m. To assess the functionality of these engineered constructs, murine L cells, which are Qa-1 deficient, were transfected with each. Expression levels were measured via western blot and flow cytometry. Functionality was assessed in a CFSE (carboxyfluorescein succinimidyl ester) based CTL killing assay.

Results – Results from SDS-PAGE experiments determined that two transgenes were expressed and appropriately N-glycosylated. These two transgenes were Tg 2 and Tg 26. Further, flow cytometry experiments demonstrated that these two transgenes were expressed on the cell surface. CFSE killing assays demonstrated that both Tg 2 and Tg 26 permitted target cell lysis in a peptide concentration dependent and effector cell dependent manner. We selected Tg 2, on the basis of superior expression and CTL recognition, for transgenic development. Unfortunately, in a developing *in vivo* murine system a CD8+ T cell phenotype was not observed.

Conclusions – The development of a scQa-1^b transgenic mouse model in theory provides a unique tool to study nonclassical, Qa-1 dependent T cell selection and the phenotypes of uncommon CD8+, Qa-1 restricted peripheral T cells. *In vitro* assays validated robust transgene expression and functional CTL recognition. However, the ability to detect peripheral CD8+ T cells *in vivo* failed. These results suggest that different genetic or protein regulatory mechanisms could be present during murine development that were not accounted for.

INTRODUCTION

MHC class I molecules serve as sensors of a cell's internal environment. During times of intracellular infection, tumorigenesis, and stress MHC I molecules are capable of alerting lymphocytes to dispose of damaged cells. Structurally, MHC I molecules are comprised of three n-terminal domains referred to as α1, α2, and α3 followed by a transmembrane domain and a c-terminal domain that extends into the cytoplasm of the cell. This structure is referred to as the heavy chain and associates noncovalently with beta-2 microglobulin (β2m), also referred to as the light chain. A key component of MHC class I's ability to identify damaged cells resides in between the α1 and α2 domains where short peptide segments ranging from eight to ten amino acids can bind the groove.[89] The presence of this peptide, MHC I, and β2m form a trimer on the cell surface that serves as a ligand for the T Cell Receptor (TCR) and certain natural killer (NK) cell receptors.

Within the MHC class I family there exists a division between Ia and Ib molecules. Classical MHC Ia (MHC Ia) molecules, which include H-2K, D, and L, are defined by extensive allelic variability. On the other hand nonclassical MHC Ib (MHC Ib) molecules are characterized by incredibly low polymorphism. Qa-1 belongs to the MHC Ib subgroup having only four known alleles.[78] Another intriguing feature of Qa-1 is that it largely displays a single peptide within its' hydrophobic binding pocket. This peptide is derived from the proteasome processed signal sequence of the MHC Ia molecules H-2D and H-2L and requires TAP (transporter associated with antigen processing) dependent transport into the endoplasmic reticulum (ER). This peptide has been named the Qa-1 determinant modifier, Qdm, and is composed of the nonameric amino acid sequence AMAPRTLLL. It has been calculated from peptide elution studies that Qdm makes up 70% of the total peptide content of Qa-1.[79] It is also worth noting that Qa-1 is the ortholog to the human HLA-E molecule. Despite only 73% amino acid sequence homology, HLA-E typically presents a similar Qdm peptide

with the sequence VMAPRTLLL. HLA-E is also non-polymorphic having only two known alleles in the human population.[78] Functionally these orthologs play similar roles in the murine and human immune system, highlighting the translational appeal of studying Qa-1.

During T cell development positive and negative selection are mediated by interactions between the T cell TCR and MHC class I. Primarily, studies on T cell development and TCR restriction have focused on the role of the highly polymorphic, classical MHC Ia molecules. However, only recently has the focus shifted to the role being played by nonclassical MHC Ib molecules during T cell development. It has been demonstrated that classical MHC Ia selection occurs on thymic epithelial cells (TECs), while non-classical MHC Ib selection can be orchestrated by both TECs and hematopoietic cells (HCs).[74,75] Intriguingly, studies have shown that MHC Ib-restricted (nonclassical) CD8+ T cells have a distinct "innate-like" phenotype, in comparison to MHC Ia-restricted (classical) CD8+ T cells. This innate-like phenotype is defined by high levels of CD44 expression on naïve, murine T cells[76], and the ability to rapidly produce cytokines upon in vitro stimulation. Further, MHC Ib restricted, CD8+ T cells have been observed to mount a quicker immune response to *Listeria monocytogenes* (LM).[76,77]

Additionally, there are a number of reports suggesting that Qa-1 restricted αβ T cells may play an important regulatory role. The observance that injecting naïve rats with an irradiated (or mitomycin C treated), myelin basic protein specific, CD4+ T cell line protects these rats from the induction of experimental allergic encephalomyelitis (EAE) has opened the door for further inquiry. This therapeutic strategy has been termed T cell vaccination (TCV). Studies of TCV in humans and rats have identified a CD8+ T cell population that specifically recognizes the pathogenic, vaccine T cells.[94,95] Work from the laboratory of Leonard Chess's lab has identified Vβ8-specific Qa-1-restricted CD8+ T cells that are induced by TCV and capable of lysing a CD4+ T cell clone that expresses Vβ8.[96]

Recently, new observations have suggested that Qa-1 restricted CD8+ regulatory cells (CD8+ Tregs) monitor follicular T helper cells (Tfh). Using a genetic model to disrupt TCR/CD8 recognition of Qa-1, work by Kim and colleagues uncovered the development of a systemic-lupus-erythematosus-like autoimmune disease driven by autoantibodies.[97] Moreover, in a mouse model of collagen-induced arthritis these CD8+ Tregs were observed to eliminate autoreactive Tfh and Th17 cells, thereby inhibiting disease development.[98]

Here we have outlined a strategy for a unique mouse model to elucidate the role of Qa-1 dependent, nonclassical T cell selection during development. This scQa-1^b; β2m -/- model also enables us to interrogate the phenotypes of Qa-1 restricted, peripheral CD8+ T cells. Such work will elucidate the prospects of the presence of an innate-like CD8+ T cell population and also CD8+ Tregs.

METHODS

scQa-1^{b}cloning

Three single-chain Qa-1^b DNA constructs were cloned, to assess CD8+ T cell development. The single-chain nomenclature refers to the insertion of a short peptide linker that covalently links the heavy chain Qa-1^b to β2-microglobulin (β2m) for appropriate expression on the cell surface and antigen presentation. The prefix Tg is used to identify these constructs since they are potential transgenes. Tg 2 was driven by the H-2D^d promoter, followed by β2m^b cDNA, a 20 amino acid linker composed of a cluster of four glycine molecules followed serine repeated four times $(G_4S)_4$, next to the genomic sequence of Qa-1^b. Tg 26 was driven by the H-2K^b promoter, followed by an intronic sequence, β2m^b cDNA, a 15 amino acid linker composed of four glycine molecules followed serine repeated three times $(G_4S)_3$, next to the cDNA sequence of Qa-1^b. Tg30 was driven by the H2-D^d promoter, followed by β2m^b cDNA, a 15 amino acid linker composed of four glycine molecules followed serine repeated three times $(G_4S)_3$, next to the genomic sequence of Qa-1^b. These constructs are illustrated in Figure 4.2.

Transfection of scQa-1^{b}in L cells

The murine, adherent fibroblast L cell line (tk-) (Ltk-) was used for target cells since they do not express endogenous Qa-1. Cells were transfected with the scQa-1^b constructs via electroporation. In summary, $10x10^6$ cells were electroporated at a voltage of 950 , capacitance of 25 µFD, and overall field strength of 2,375, in calcium and magnesium free Hank's Buffered Saline Solution with 20 ug of each DNA construct. A Bio-Rad gene pulser was used for the electroporation. Serial diluton in a 96 well plate allowed for the outgrowth of single clones. After transfection cells

were maintained in media consisting of DMEM, 10% FBS, P/S/Q, and G418 to allow for drug resistant selection.

Detection of scQa-1^b expression

Expression levels were determined using two techniques: western blotting and flow cytometry. For western blotting scQa-1^b transfected L cell clones (Tg 2, Tg 26, and Tg 30) were lysed and denatured in sample buffer with 2-merccaptoethanol followed by 3 minutes of boiling. Lysates were run on 10% SDS-PAGE gels and transferred to nitrocellulose. The 6A8.6F10.1A6 antibody at a concentration of 1:500 was used for primary detection. Anti-mouse IgG horseradish peroxidase conjugated antibody at a concentration of 1:10,000 was used for secondary detection and visualized using chemiluminescence (ThermoFisher, Pierce SuperSignal West Pico).

CFSE based killing assay

Target cells and bystander cells were labeled at two different concentrations of CFSE. $CFSE_{Low}$ targets were labeled at a final concentration of 0.42 uM and $CFSE_{high}$ bystander cells were labeled at a concentration of 5 uM. In brief, a description of the labeling process. A stock solution of CFSE (Biolegend, 422701) was diluted in PBS and incubated with the relevant cell line for 10 minutes at 37°C, while protected from light (total volume was equal to 1 ml). Staining was quenched by adding 10 ml of ice cold DMEM with 10% FBS. Cells were washed by pelleting via centrifugation, washed 1x with PBS, and then resuspended in RPMI 1640 with 10% FBS plus P/S/Q supplements. Cells were plated at the appropriate cell count in a 96 well round bottom dish. Then these target and bystander cells were incubated with the appropriate CTL clone, D5D2 or 524.D11, and the plates were briefly centrifuged to promoted cell-to-cell contact. The CTL mediated killing incubation took place for 4 hours at 37°C in a tissue culture incubator. After this incubation cells were harvested for

analysis via flow cytometry. The plates were centrifuged to pellet targets, bystanders, and CTL clones. Media was next aspirated off. Since L cells are adherent, the cells were incubated with Cellstripper for 15 minutes. Cells were next stained with TO-PRO-3 iodide (ThermoFisher, T3605) at a concentration of 1 ul per 100,000 cells. Further, anti-CD8 conjugated PE antibody was included to gate out the CD8+ CTL clones. This staining cocktail was incubated with cells for 25 minutes. After, cells were washed 1x and analyzed on a FACS Calibur.

Transgenic development

Transgenic mouse model development was conducted by the gene core at the University of Utah in collaboration with Dr. Peter Jensen's laboratory. Tg 2 was linearized and injected into the pronucleus of a C57BL/6 zygote, prior to breeding to β2m -/- mice.

RESULTS

The design of a single-chain Qa-1^b molecule

To understand the role that Qa-1 serves during T cell development, we have designed a transgenic mouse model that expresses Qa-1 as the sole MHC I molecule. An illustration of this model is presented in Figure 4.1. To start, three DNA constructs that covalently link β2m^b to the genomic (gDNA) or complementary (cDNA) sequence of Qa-1^b via an amino acid linker were designed. We refer to these molecules as scQa-1^b. The amino acid linker is composed of repeats of four glycine and one serine (G_4S). A detailed map of these potential transgenes (Tg) can be seen in Figure 4.2.

These three constructs have slightly varied components in the promoter, linker, and Qa-1^b gene. Tg 2 and Tg 30 are both driven by the H2-D^d promoter and contain the genomic sequence of Qa-1^b. They also differ in the size of their amino acid (aa) linker. Tg 2 has a 20 aa linker and Tg 30 has a 15 aa linker. This linker or spacer is included to create physical distance and foster a β2m^b:Qa-1 interaction that mirrors the wild-type interaction. Tg 26 is the most divergent of the constructs utilizing the H-2K^b promoter, a 15 aa linker, and a Qa-1^b cDNA sequence. Additionally, this sequence contains an intron aimed at enhancing expression in the transgenic system.[99]

Validation of scQa-1b expression

To determine that the scQa-1^b molecules could be expressed and remain intact, we stably transfected the murine L (tk-) cell line (Ltk) with each transgene. This fibroblast cell line is Qa-1 deficient making them a choice cell line for scQa-1^b transfection. Clones were isolated via limiting dilution in a ninety-six well plate and expression was assessed by SDS-PAGE and flow cytometry (figure 4.3). The wild-type Qa-1 migrates at 44 kDa and is detected in wild-type murine PBMC lysate;

while the scQa-1^b constructs migrate at a molecular weight of 60 kDa, representing the convalent linkage of $\beta 2m^b$ via an amino acid linker. The higher and fainter band represents the mature N-glycosylated form (figure 4.3A). Two clones for each transgene with strong expression were observed for Tg 2 and Tg 26, while Tg 30 levels were nearly undetectable by western blot.

Surface expression of scQa-1^b was tested by flow cytometry. Only one clone, 30E, failed to consistently express scQa-1^b on the cell surface. The remaining clones had noticeable surface expression as determined by histogram analysis and mean fluorescence intensity. These results are displayed in Figure 4.3B and Figure 4.3C.

Functional recognition of scQa-1^b CTL clones

To determine if the scQa-1^b was functional as an antigen presentation molecule, we optimized a CFSE based killing assay. First, target cells (scQa-1^b+) and bystander cells (scQa-1^b(-)) were labeled with CFSE at two different concentrations. Target cells were $CFSE_{Low}$ $[0.42uM]_{final}$ and bystanders $CFSE_{High}$ $[5uM]_{final}$. This 10-fold difference in CFSE labeling concentrations produced two distinct, clearly labeled cell populations that could be easily detected and seperated by flow cytometry analysis. To assess CTL dependent recognition we incubated target cell clones expressing the appropriate scQa-1^b transgene, Qa-1 deficient bystander cells (Ltk), and the CTL clone D5D2 in 96 well plates. D5D2 is a Qdm specific CTL clone that is Qa-1^b restricted and recognizes endogenous Qdm+ (AMAPRTLLL) in L cells.[100] After a 4 hour incubation, cells were harvested and assessed via flow cytometry. The CTLs (CD8+) and dead cells (CFSE negative) were gated out. Cell killing was measured by three readouts: loss of CFSE, reduced forward scatter (FSC), and TO-PRO-3 staining; a maker of cell death. D5D2 recognition and killing was dose dependent on the basis of the number of effector CTLs incubated with targets and bystanders (figure 4.4A). Lysis of Tg 2L and Tg 26H, was comparable to wild-type Qa-1^b present on Lg37 cells. Minimal cell death was observed in the Ltk –

Ltk treatment (bystander-bystander) showing the assay's capability of detecting antigen-sepecific CTL activity. The CFSE labeling, loss of CFSE, reduced FSC, and TO-PRO-3 positivity are completely concordant with one another as demonstrated in Figure 4.5, at a 5:1 effector to target cell ratio (E:T).

Further, to assess the sensitivity of our CFSE killing assay as well as the sensitivity of scQa-1^b recognition, target cells were incubated with the 524.D11 CTL clone. This CTL clone is Qa-1^b restricted and dependent on the Qdm^k (AM**V**PRTLL) peptide which is not endogenously present in L cells. Therefore, experiments were performed as described above, with the addition of Qdm^k peptide titrated into the assay at concentrations of 0 nm, 1 nm, 10 nm, and 100 nm. The dose response curve observed in this assay (Figure 4.4B) confirmed that both our scQa-1^b clones, Tg 2L and Tg26H, could be detected with great sensitivity in a peptide dependent manner.

As a result of its' high level expression of the mature N-glycosylated form of scQa-1^b and the observance that CTL recognition mirrored wild-type Qa-1^b, Tg 2 was selected for transgenic development.

Transgenic scQa-1^b; β2m -/- mouse model development

To develop the B6-scQa-1^b; β2m -/- transgenic line, Tg 2 DNA was purified using a cesium chloride gradient. Next, it was linearized and injected into the pronucleus of C57BL/6 (B6) zygotes. Transgene positive progeny were identified using PCR and positive offspring were then bred onto congenic B6-β2m -/- background. As illustrated in Figure 4.1 this strategy creates a strain where Qa-1^b is the sole MHC I molecule expressed. When B6-scQa-1^b; β2m -/- progency were available we analyzed the mice for the presence of peripheral CD8+ T cells and CD8+ thymocytes. To our surprise

flow cytometry experiments demonstrated no CD8+ T cells in the periphery or thymus (data not shown).

DISCUSSION

Here we outline the development of a scQa-1^b; β2m-/- mouse model to assess the role played by the nonclassical MHC Ib molecule Qa-1^b in T cell development and function. The design of this reductionist system ensures that Qa-1 is the sole MHC I molecule expressed. In *in vitro* studies we determined that the scQa-1^b expression vector could be expressed, trafficked to the cell surface, and functionally recognized by multiple CTL clones. Two out of three of our designed Tg constructs were validated in these assays. Tg 2 was selected for scQa-1^b mouse model development since it demonstrated strong, consistent expression and CTL recognition that paralleled wild-type Qa-1^b. Unfortunately, after scQa-1^b transgenic development and breeding onto the β2m -/- background, a CD8+ T cell phenotype was not observed. CD8+ T cells were not detected in the periphery nor the thymus. Consequently, we were unable to study Qa-1 based nonclassical T cell development, the potential presence of a Qa-1 restricted "innate-like" cell subset, nor provide greater clarity to the role of Qa-1 restricted CD8+ Tregs.

The inability to translate our *in vitro* observances into an *in vivo* system may have been caused by a number of factors. The overriding theme is that in an *in vivo* mouse model there may exist genetic or protein regulatory elements, that were absent in transformed L cells. For example, the thymic epithelial cells (TECs) and hematopoietic cells (HCs) that mediate T cell selection may express Qa-1^b not at all, at lower levels, or perhaps only under specific circumstances. Epigenetic factors may have also contributed to the absence of our hypothesized phenotype. It is possible that during pronuclear injection or breeding that the transgene was localized to a silenced chromosomal region. Moreover, it is possible that our engineered construct was silenced as a result of its' non-native sequence.

Despite this unsuccessful attempt, during this investigation we optimized a non-radioactive CFSE based CTL killing assay utilizing flow cytometry. In this assay, target cells and bystander cells were labeled at two different CFSE concentrations, 0.42 uM and 5 uM, and together they were incubated with a CTL clone. CTL killing was measured by flow cytometry. We used TO-PRO-3 iodide as a measure of cell viability, and also observed that cell death correlated with reduced forward scatter (FSC) and loss of CFSE staining. On the basis of this finding we suggest that target cells always be labeled $CFSE_{low}$. This ensures that dead bystander cells do not contaminate the $CFSE_{low}$ labeled target population. This assay was sensitive enough to display a dose response curve dependent on the number of effector CTLs and also the concentration of peptide.

Historically, the gold standard measure of CTL mediated killing is assessed by a chromium (^{51}Cr) release assay. This assay, developed by Brunner and colleagues[101], uses the release of the radioactive ^{51}Cr isotope into the supernatant as a measure of cell death. This assay has proven to be effective, but does have some drawbacks which include the hazard, cost, cumbersome nature of radioactivity usage, the difficulties of labeling target cells with ^{51}Cr, and the spontaneous release of ^{51}Cr that can lead to high background levels. Our CFSE based CTL killing assay, circumvents these challenges and provides a much needed update to a classic and useful technique.

Additionally, using flow cytometry for this assay allows for a multiparametric analysis of CD8+ T cells. While not necessary for the studies described here, it is possible to expand on CD8+ T cell phenotype by coupling CTL lysis with other markers such as intracellular cytokine levels (i.e. IFN-γ, IL-17, TNF-α, etc.), upregulated transcription factors (T-bet, Eomes, HELIOS, etc.), and additional cell surface molecules (i.e. PD-1, VISTA, CTLA-4, etc).

We note that in our studies we utilized an immortalized, murine cell line with a restricted MHC haplotype. However, recently Mbitikon-Kobo and colleagues also developed a CFSE based

killing assay using human PBMCs.[102] Intelligently, they selected autologous B cells as the antigen-presenting target cells and used polyclonal CD8+ T cells. Further, they enriched for the peptide specific CD8+ CTLs by incorporating peptide:MHC (pMHC) tetramers. We feel that this was a strategically well designed assay, and can be technically improved by incorporating our CFSE labeling and E:T incubation methods. Thus creating more clearly labeled target and bystander cell populations, while also reducing the overall incubation time of the experiment.

The study of the role played by nonclassical MHC Ib in T cell development is an emerging and overlooked niche of the immune system. Multiple groups have made key insights, suggesting these T cells do have important immunological roles, but remain understudied. It is our hope that the strategies outlined in our scQa-1^b;β2m -/- mouse model can be used by others to study Qa-1, as well as the other fifteen murine and eleven human nonclassical MHC Ib molecules. We also hope that the numerous advantages outlined by our CFSE, flow cytometry based CTL killing assay can replace the ^{51}Cr release assay.

FIGURES

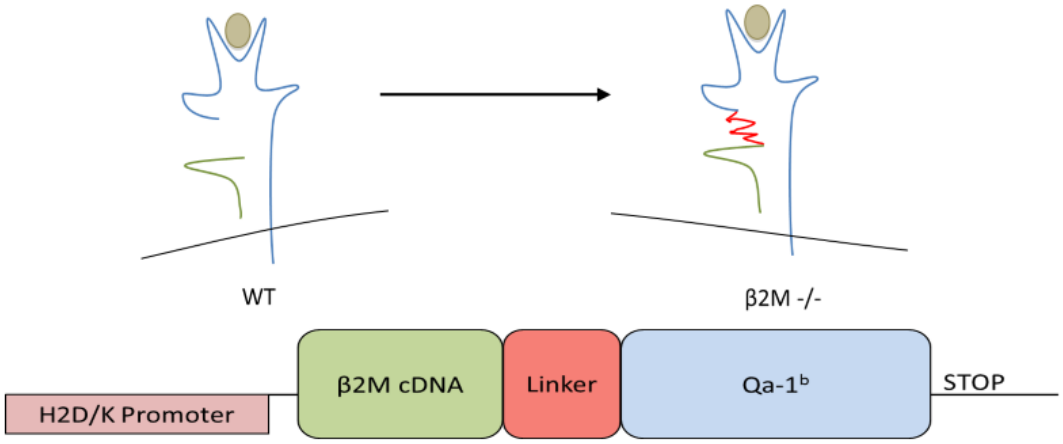


Figure 4.1 Design of the scQa-1^b molecule at the genetic and protein level

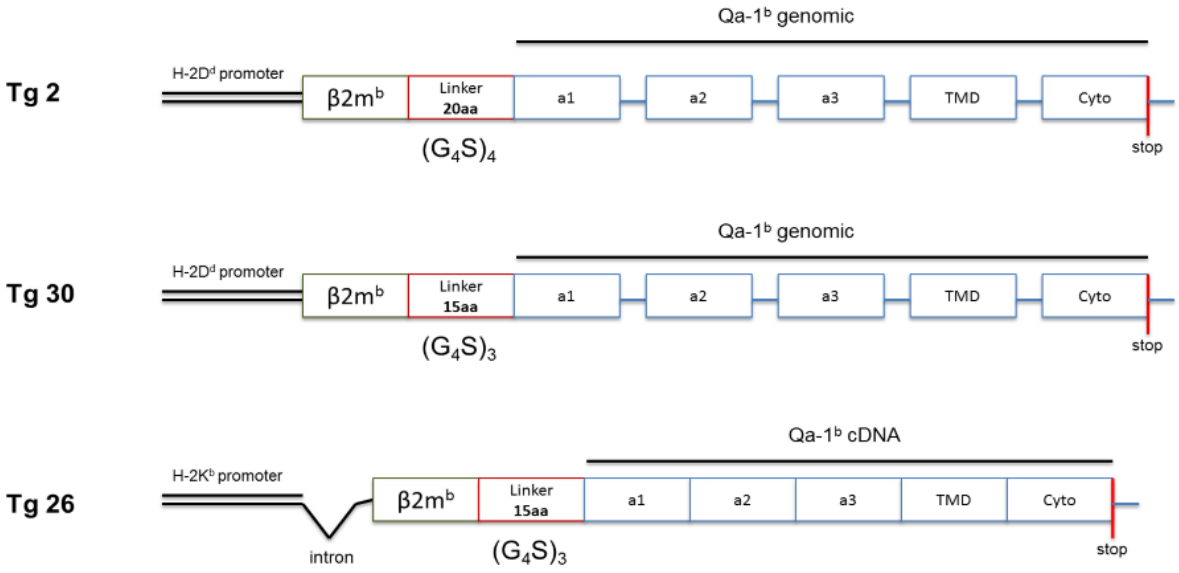


Figure 4.2 Diagrams of the scQa-1^b DNA constructs

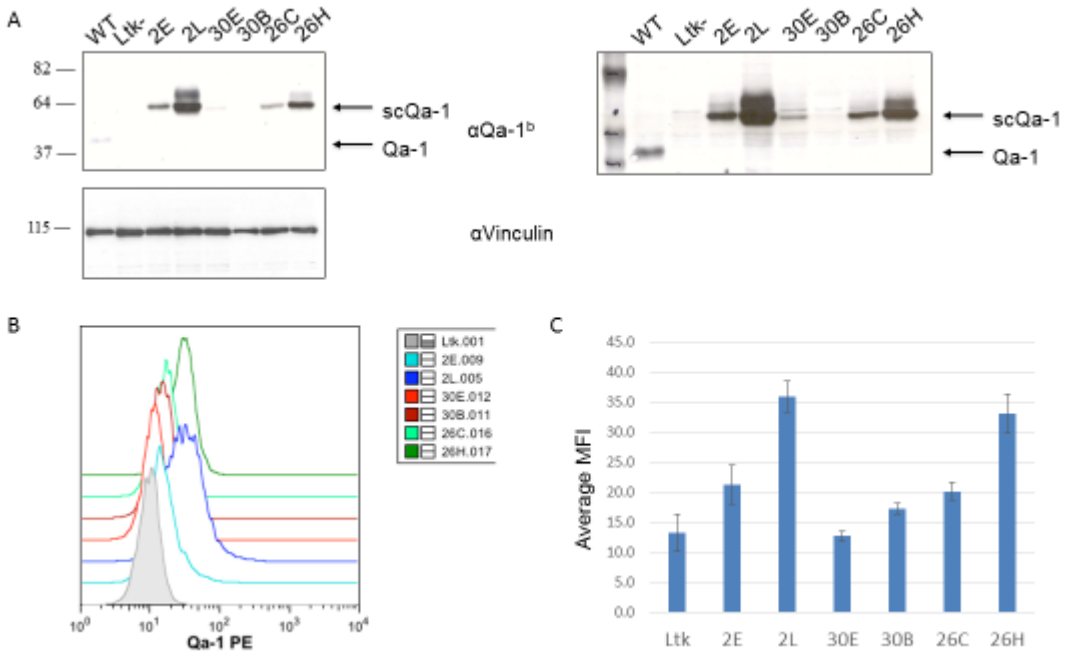


Figure 4.3 Expression of the scQa-1^{b} molecules as assessed by SDS-PAGE and flow cytometry. (A) L (tk-) cells were stably transfected with the following constructs g37 (wild-type Qa-1), Tg 2, Tg30, and Tg 26. Clones were isolated via limiting dilution and Qa-1 expression was determined via SDS-PAGE. Clone 2L and 26H demonstrated robust expression. A longer exposure can be seen on the right and shows wild-type Qa-1 expression (B) Flow cytometry histogram analysis identifies robust surface expression on clones 2L and 26H. (C) Quantitated mean fluorescence intensity (MFI) of isolated clones presented with the standard error of the mean.

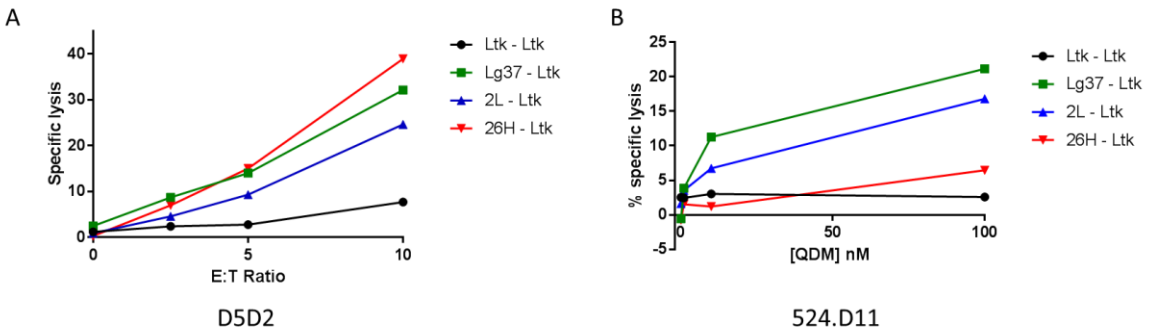


Figure 4.4 scQa-1^b is functionally recognized by effector CTL clones in an effector cell and peptide dependent manner (A) Tg 2, Tg 26, and g37 (wild-type) expressed by stably transfected L cells are recognized as targets and lysed by D5D2 in an effector:target cell dependent fashion at E:T ratios over the range of 0, 2.5, 5, and 10. (B) Tg 2, Tg 26, and g37 in L cells is recognized in a peptide dependent manner using Qdm^k (AMVPRTLL) at concentrations of 0 nm, 1 nm, 10 nm, and 100 nm and the 524.D11 CTL clone.

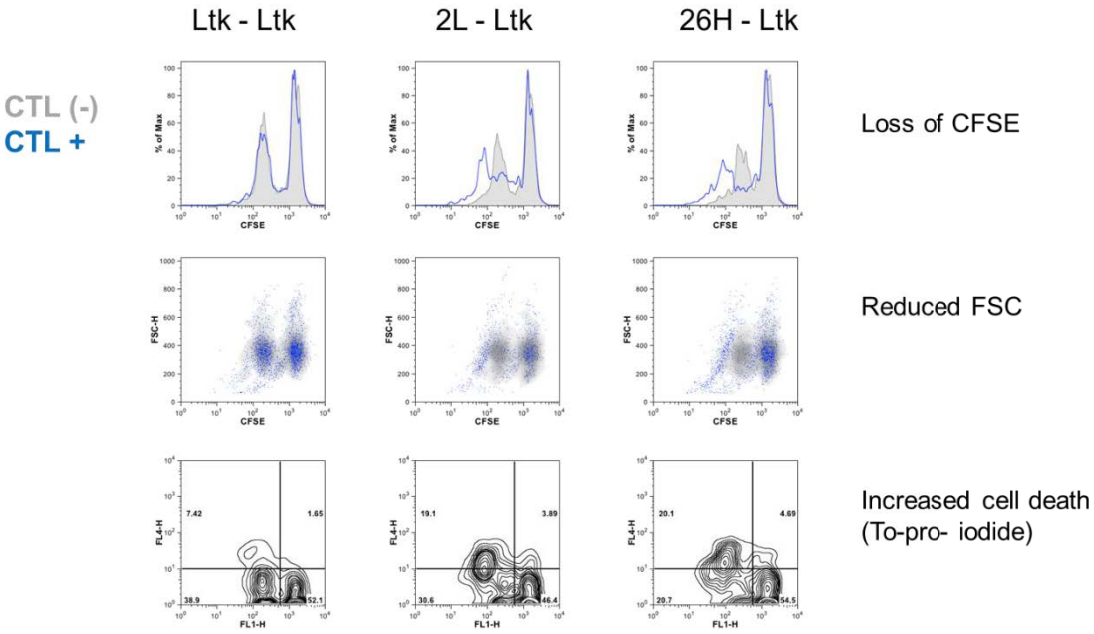


Figure 4.5 *In vitro* CFSE based CTL killing assay demonstrates 3 readouts of cell death: loss of CFSE, reduced FSC, and TO-PRO-3 iodide positive staining. Target cells were incubated with D5D2 at an E:T of 5:1. Ltk cells are left untouched by D5D2, however Tg 2L clone and Tg 26H clone are specifically targeted for lysis, even in the presence of L (tk-) cell bystanders.

REFERENCES CITED

1. Dougados, M. & Baeten, D. Spondyloarthritis. *The Lancet* **377,** 2127–2137 (2011).
2. Khan, M. A. Update on Spondyloarthropathies. *Ann. Intern. Med.* **136,** 896 (2002).
3. Moll, J. M. H. B. S., Haslock, I. M. D., Macrae, I. F. M. B. & Wright, V. M. D. ASSOCIATIONS BETWEEN ANKYLOSING SPONDYLITIS, PSORIATIC ARTHRITIS, REITER'S DISEASE, THE INTESTINAL ARTHROPATHIES, AND BEHCET'S SYNDROME. *Medicine (Baltimore)* **53,** 343–364 (1974).
4. Feldtkeller, E., Khan, M., Heijde, D. van der, Linden, S. van der & Braun, J. Age at disease onset and diagnosis delay in HLA-B27 negative vs. positive patients with ankylosing spondylitis. *Rheumatol. Int.* **23,** 61–66 (2003).
5. Braun, J. & Sieper, J. Ankylosing spondylitis. *The Lancet* **369,** 1379–1390 (2007).
6. Thomas, G. P. & Brown, M. A. Genetics and genomics of ankylosing spondylitis. *Immunol. Rev.* **233,** 162–180 (2010).
7. Tsui, F. W., Tsui, H. W., Akram, A., Haroon, N. & Inman, R. D. The genetic basis of ankylosing spondylitis: new insights into disease pathogenesis. *Appl. Clin. Genet.* **7,** 105 (2014).
8. Hammer, R. E., Maika, S. D., Richardson, J. A., Tang, J.-P. & Taurog, J. D. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human β2m: An animal model of HLA-B27-associated human disorders. *Cell* **63,** 1099–1112 (1990).
9. Taurog, J. D. *et al.* Spondylarthritis in HLA–B27/human β2-microglobulin–transgenic rats is not prevented by lack of CD8. *Arthritis Rheum.* **60,** 1977–1984 (2009).
10. May, E. *et al.* CD8αβ T Cells Are Not Essential to the Pathogenesis of Arthritis or Colitis in HLA-B27 Transgenic Rats. *J. Immunol.* **170,** 1099–1105 (2003).
11. Taurog, J. D. *et al.* The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J. Exp. Med.* **180,** 2359–2364 (1994).

12. (igas), I. G. of A. S. C. Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci. *Nat. Genet.* **45,** 730–738 (2013).

13. (tasc), T. A.-A.-A. S. C. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. *Nat. Genet.* **42,** 123–127 (2010).

14. Kellgren, J. H., Jeffrey, M. R. & Ball, J. *The epidemiology of chronic rheumatism: a symposium*. **2,** (FA Davis Company, 1963).

15. Bennett, P. H. & Burch, T. A. in *Population studies of the rheumatic diseases* 305–313 (Excerpta Medica, Amsterdam, 1968).

16. Linden, S. V. D., Valkenburg, H. A. & Cats, A. Evaluation of Diagnostic Criteria for Ankylosing Spondylitis. *Arthritis Rheum.* **27,** 361–368 (1984).

17. Huppertz, H.-I., Voigt, I., Müller-Scholden, J. & Sandhage, K. Cardiac Manifestations in Patients with HLA B27-Associated Juvenile Arthritis. *Pediatr. Cardiol.* **21,** 141–147 (2000).

18. Kim, T. H., Jung, S. S., Sohn, S. J., Park, M. H. & Kim, S. Y. Aneurysmal Dilatation of Ascending Aorta and Aortic Insufficiency in Juvenile Spondyloarthropathy. *Scand. J. Rheumatol.* **26,** 218–221 (1997).

19. Roldan, C. A., Chavez, J., Wiest, P. W., Qualls, C. R. & Crawford, M. H. Aortic root disease and valve disease associated with ankylosing spondylitis. *J. Am. Coll. Cardiol.* **32,** 1397–1404 (1998).

20. Bergfeldt, L. HLA-B27-associated cardiac disease. *Ann. Intern. Med.* **127,** 621–629 (1997).

21. Forsblad-d'Elia, H., Wallberg, H., Klingberg, E., Carlsten, H. & Bergfeldt, L. Cardiac conduction system abnormalities in ankylosing spondylitis: a cross-sectional study. *BMC Musculoskelet. Disord.* **14,** 237 (2013).

22. Klingberg, E. *et al.* Aortic Regurgitation Is Common in Ankylosing Spondylitis: Time for Routine Echocardiography Evaluation? *Am. J. Med.* **128,** 1244–1250.e1 (2015).

23. Blumberg, B. S., Bunim, J. J., Calkins, E., Pirani, C. L. & Zvaifler, N. J. NOMENCLATURE AND CLASSIFICATION OF ARTHRITIS AND RHEUMATISM (TENTATIVE) ACCEPTED BY THE AMERICAN RHEUMATISM ASSOCIATION. *Bull. Rheum. Dis.* **14,** 339 (1964).

24. Haroon, M. & FitzGerald, O. Psoriatic arthritis: complexities, comorbidities and implications for the clinic. *Expert Rev. Clin. Immunol.* **12,** 405–416 (2016).

25. Esposito, M. *et al.* Efficacy and Safety of Subcutaneous Anti-Tumor Necrosis Factor-Alpha Agents, Etanercept and Adalimumab, in Elderly Patients Affected by Psoriasis and Psoriatic Arthritis: An Observational Long-Term Study. *Dermatology* **225,** 312–319 (2012).

26. Versini, M., Jeandel, P.-Y., Rosenthal, E. & Shoenfeld, Y. Obesity in autoimmune diseases: Not a passive bystander. *Autoimmun. Rev.* **13,** 981–1000 (2014).

27. Haque, N., Lories, R. J. & Vlam, K. de. Comorbidities Associated with Psoriatic Arthritis Compared with Non-psoriatic Spondyloarthritis: A Cross-sectional Study. *J. Rheumatol.* **43,** 376–382 (2016).

28. Gelfand, J. M. *et al.* Epidemiology of psoriatic arthritis in the population of the United States. *J. Am. Acad. Dermatol.* **53,** 573.e1-573.e13 (2005).

29. Winchester, R. *et al.* HLA associations reveal genetic heterogeneity in psoriatic arthritis and in the psoriasis phenotype. *Arthritis Rheum.* **64,** 1134–1144 (2012).

30. Bowes, J. *et al.* Evidence to support IL-13 as a risk locus for psoriatic arthritis but not psoriasis vulgaris. *Ann. Rheum. Dis.* **70,** 1016–1019 (2011).

31. Sukhov, A., Adamopoulos, I. E. & Maverakis, E. Interactions of the Immune System with Skin and Bone Tissue in Psoriatic Arthritis: A Comprehensive Review. *Clin. Rev. Allergy Immunol.* **51,** 87–99 (2016).

32. Taylor, W. *et al.* Classification criteria for psoriatic arthritis: Development of new criteria from a large international study. *Arthritis Rheum.* **54,** 2665–2673 (2006).

33. Tillett, W. *et al.* The ClASsification for Psoriatic ARthritis (CASPAR) Criteria – A Retrospective Feasibility, Sensitivity, and Specificity Study. *J. Rheumatol.* **39,** 154–156 (2012).

34. Gladman, D. D., Chandran, V., Thavaneswaran, A. & Zummer, M. Psoriatic Arthritis in Canadian Clinical Practice: The PsA Assessment in Rheumatology. *J. Rheumatol.* **39,** 1850–1853 (2012).

35. Gladman, D. D., Shuckett, R., Russell, M. L., Thorne, J. C. & Schachter, R. K. Psoriatic arthritis (PSA)--an analysis of 220 patients. *Q. J. Med.* **62,** 127–141 (1987).

36. Gladman, D. D., Anhorn, K. A., Schachter, R. K. & Mervart, H. HLA antigens in psoriatic arthritis. *J. Rheumatol.* **13,** 586–592 (1986).

37. Conigliaro, P. *et al.* Autoantibodies in inflammatory arthritis. *Autoimmun. Rev.* doi:10.1016/j.autrev.2016.03.003

38. A, S. *et al.* Relative value of erythrocyte sedimentation rate and C-reactive protein in assessment of disease activity in ankylosing spondylitis. *J. Rheumatol.* **26,** 980–984 (1999).

39. Rosen, A. & Casciola-Rosen, L. Autoantigens as Partners in Initiation and Propagation of Autoimmune Rheumatic Diseases. *Annu. Rev. Immunol.* **34,** 395–420 (2016).

40. Laloux, L. *et al.* Immunohistological study of entheses in spondyloarthropathies: comparison in rheumatoid arthritis and osteoarthritis. *Ann. Rheum. Dis.* **60,** 316–321 (2001).

41. Cañete, J. D. *et al.* Ectopic lymphoid neogenesis in psoriatic arthritis. *Ann. Rheum. Dis.* **66,** 720–726 (2007).

42. Takemura, S. *et al.* Lymphoid Neogenesis in Rheumatoid Synovitis. *J. Immunol.* **167,** 1072–1080 (2001).

43. Kang, Y. M. *et al.* CD8 T Cells Are Required for the Formation of Ectopic Germinal Centers in Rheumatoid Synovitis. *J. Exp. Med.* **195,** 1325–1336 (2002).

44. Manzo, A. *et al.* Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. *Eur. J. Immunol.* **35,** 1347–1359 (2005).

45. Weyand, C. M. & Goronzy, J. J. Ectopic Germinal Center Formation in Rheumatoid Synovitis. *Ann. N. Y. Acad. Sci.* **987,** 140–149 (2003).

46. Clinical manifestations and diagnosis of psoriatic arthritis. *UpToDate* Available at: http://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-psoriatic-arthritis?source=search_result&search=CASPAR&selectedTitle=1%7E1. (Accessed: 24th May 2016)

47. Psoriatic arthritis: complexities, comorbidities and implications for the clinic - 1744666X.2016.1139453.

48. van der Helm-van Mil, A. H., Verpoort, K. N., Breedveld, F. C., Toes, R. E. & Huizinga, T. W. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res. Ther.* **7,** R949 (2005).

49. Casciola-Rosen, L. & Mammen, A. L. Myositis autoantibodies. *Curr. Opin. Rheumatol.* **24,** 602–608 (2012).

50. Johnson, C. *et al.* Clinical and pathologic differences in interstitial lung disease based on antisynthetase antibody type. *Respir. Med.* **108,** 1542–1548 (2014).

51. Yaniv, G. *et al.* A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. *Autoimmun. Rev.* **14,** 75–79 (2015).

52. Guis, S. *et al.* TWEAK-binding autoantibodies are generated during psoriatic arthritis and are not influenced by anti-TNF therapy. *J. Transl. Med.* **14,** 185 (2016).

53. Maejima, H. *et al.* Moesin and Stress-Induced Phosphoprotein-1 Are Possible Sero-Diagnostic Markers of Psoriasis. *PLOS ONE* **9,** e101773 (2014).

54. Chimenti, M. S. *et al.* Auto-reactions, autoimmunity and psoriatic arthritis. *Autoimmun. Rev.* **14,** 1142–1146 (2015).

55. Dalmády, S. *et al.* Higher Levels of Autoantibodies Targeting Mutated Citrullinated Vimentin in Patients with Psoriatic Arthritis Than in Patients with Psoriasis Vulgaris. *Clin. Dev. Immunol.* **2013,** (2013).

56. Dolcino, M. *et al.* Crossreactive Autoantibodies Directed against Cutaneous and Joint Antigens Are Present in Psoriatic Arthritis. *PLOS ONE* **9,** e115424 (2014).

57. Silvy, F. *et al.* Antinuclear Antibodies in Patients with Psoriatic Arthritis Treated or Not with Biologics. *PLOS ONE* **10,** e0134218 (2015).

58. Targoff, I. N. Update on myositis-specific and myositis-associated autoantibodies. *Curr. Opin. Rheumatol.* **12,** 475–481 (2000).

59. Arnett, F. C., Hirsch, T. J., Bias, W. B., Nishikai, M. & Reichlin, M. The Jo-1 antibody system in myositis: relationships to clinical features and HLA. *J. Rheumatol.* **8,** 925–930 (1981).

60. Bernstein, R. M. *et al.* Anti-Jo-1 antibody: a marker for myositis with interstitial lung disease. *Br. Med. J. Clin. Res. Ed* **289,** 151–152 (1984).

61. Fiorentino, D. F. *et al.* PUF60: a prominent new target of the autoimmune response in dermatomyositis and Sjögren's syndrome. *Ann. Rheum. Dis.* (2015). doi:10.1136/annrheumdis-2015-207509

62. DeBenedittis, P. & Jiao, K. Alternative splicing of T-box transcription factor genes. *Biochem. Biophys. Res. Commun.* **412,** 513–517 (2011).

63. Meager, A. *et al.* Anti-interferon autoantibodies in autoimmune polyendocrinopathy syndrome type 1. *PLoS Med.* **3,** e289 (2006).

64. Meager, A. *et al.* Anti-cytokine autoantibodies in autoimmunity: preponderance of neutralizing autoantibodies against interferon-alpha, interferon-omega and interleukin-12 in patients with thymoma and/or myasthenia gravis. *Clin. Exp. Immunol.* **132,** 128–136 (2003).

65. Meyer, S. *et al.* AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies. *Cell* **166,** 582–595 (2016).

66. al, N. K., et. Positional cloning of the APECED gene. - PubMed - NCBI. Available at: https://www.ncbi.nlm.nih.gov/pubmed/?term=nagamine+1997+AND+Peterson. (Accessed: 1st November 2016)

67. Wildbaum, G., Nahir, M. A. & Karin, N. Beneficial Autoimmunity to Proinflammatory Mediators Restrains the Consequences of Self-Destructive Immunity. *Immunity* **19,** 679–688 (2003).

68. Stennard, F. A. *et al.* Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev. Biol.* **262,** 206–224 (2003).

69. Kirk, E. P. *et al.* Mutations in Cardiac T-Box Factor Gene TBX20 Are Associated with Diverse Cardiac Pathologies, Including Defects of Septation and Valvulogenesis and Cardiomyopathy. *Am. J. Hum. Genet.* **81,** 280–291 (2007).

70. Liu, C. *et al.* T-box transcription factor TBX20 mutations in Chinese patients with congenital heart disease. *Eur. J. Med. Genet.* **51,** 580–587 (2008).

71. Posch, M. G. *et al.* A gain-of-function TBX20 mutation causes congenital atrial septal defects, patent foramen ovale and cardiac valve defects. *J. Med. Genet.* **47,** 230–235 (2010).

72. Pyati, U. J., Cooper, M. S., Davidson, A. J., Nechiporuk, A. & Kimelman, D. Sustained Bmp signaling is essential for cloaca development in zebrafish. *Development* **133,** 2275–2284 (2006).

73. Dimitriou, R., Tsiridis, E. & Giannoudis, P. V. Current concepts of molecular aspects of bone healing. *Injury* **36,** 1392–1404 (2005).

74. Urdahl, K. B., Sun, J. C. & Bevan, M. J. Positive selection of MHC class Ib–restricted CD8+ T cells on hematopoietic cells. *Nat. Immunol.* **3,** 772–779 (2002).

75. Cho, H., Bediako, Y., Xu, H., Choi, H.-J. & Wang, C.-R. Positive selecting cell type determines the phenotype of MHC class Ib-restricted CD8+ T cells. *Proc. Natl. Acad. Sci.* **108,** 13241–13246 (2011).

76. Seaman, M. S., Wang, C.-R. & Forman, J. MHC Class Ib-Restricted CTL Provide Protection Against Primary and Secondary Listeria monocytogenes Infection. *J. Immunol.* **165,** 5192–5201 (2000).

77. Ploss, A., Leiner, I. & Pamer, E. G. Distinct Regulation of H2-M3-Restricted Memory T Cell Responses in Lymph Node and Spleen. *J. Immunol.* **175,** 5998–6005 (2005).

78. Strong, R. K. *et al.* HLA-E Allelic Variants CORRELATING DIFFERENTIAL EXPRESSION, PEPTIDE AFFINITIES, CRYSTAL STRUCTURES, AND THERMAL STABILITIES. *J. Biol. Chem.* **278,** 5082–5090 (2003).

79. DeCloux, A., Woods, A. S., Cotter, R. J., Soloski, M. J. & Forman, J. Dominance of a single peptide bound to the class I(B) molecule, Qa-1b. *J. Immunol.* **158,** 2183–2191 (1997).

80. Valés-Gómez, M., Reyburn, H. T., Erskine, R. A., López-Botet, M. & Strominger, J. L. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* **18,** 4250–4260 (1999).

81. Ljunggren, H.-G. & Kärre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11,** 237–244 (1990).

82. Lanier, L. L. NK CELL RECOGNITION. *http://dx.doi.org/10.1146/annurev.immunol.23.021704.115526* (2004). Available at: http://www.annualreviews.org/doi/full/10.1146/annurev.immunol.23.021704.115526?url_ver=Z

39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed&. (Accessed: 17th February 2017)

83. Lo, W.-F. *et al.* Molecular mimicry mediated by MHC class Ib molecules after infection with Gram-negative pathogens. *Nat. Med.* **6,** 215–218 (2000).

84. Bouwer, H. G. *et al.* An H2-T MHC class Ib molecule presents Listeria monocytogenes-derived antigen to immune CD8+ cytotoxic T cells. *J. Immunol.* **152,** 5352–5360 (1994).

85. Bouwer, H. G., Seaman, M. S., Forman, J. & Hinrichs, D. J. MHC class Ib-restricted cells contribute to antilisterial immunity: evidence for Qa-1b as a key restricting element for Listeria-specific CTLs. *J. Immunol.* **159,** 2795–2801 (1997).

86. Bouwer, H. G. A. *et al.* Listeria monocytogenes-Infected Hepatocytes Are Targets of Major Histocompatibility Complex Class Ib-Restricted Antilisterial Cytotoxic T Lymphocytes. *Infect. Immun.* **66,** 2814–2817 (1998).

87. Seaman, M. S., Pérarnau, B., Lindahl, K. F., Lemonnier, F. A. & Forman, J. Response to Listeria monocytogenes in Mice Lacking MHC Class Ia Molecules. *J. Immunol.* **162,** 5429–5436 (1999).

88. Bouwer, H. G. A., Barry, R. A. & Hinrichs, D. J. Lack of Expansion of Major Histocompatibility Complex Class Ib-Restricted Effector Cells following Recovery from Secondary Infection with the Intracellular Pathogen Listeria monocytogenes. *Infect. Immun.* **69,** 2286–2292 (2001).

89. Rodgers, J. R. & Cook, R. G. MHC class Ib molecules bridge innate and acquired immunity. *Nat. Rev. Immunol.* **5,** 459–471 (2005).

90. Jensen, P. E., Sullivan, B. A., Reed-Loisel, L. M. & Weber, D. A. Qa-1, a nonclassical class I histocompatibility molecule with roles in innate and adaptive immunity. *Immunol. Res.* **29,** 81–92 (2004).

91. Davies, A. *et al.* A Peptide from Heat Shock Protein 60 Is the Dominant Peptide Bound To Qa-1 in the Absence of the MHC Class Ia Leader Sequence Peptide Qdm. *J. Immunol.* **170,** 5027–5033 (2003).

92. Nagarajan, N. A., Gonzalez, F. & Shastri, N. Nonclassical MHC class Ib-restricted cytotoxic T cells monitor antigen processing in the endoplasmic reticulum. *Nat. Immunol.* **13,** 579–586 (2012).

93. Oliveira, C. C. *et al.* The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J. Exp. Med.* **207,** 207–221 (2010).

94. Zhang, J., Medaer, R., Stinissen, P., Hafler, D. & Raus, J. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. *Science* **261,** 1451–1454 (1993).

95. Sun, D., Qin, Y., Chluba, J., Epplen, J. T. & Wekerle, H. Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T–T cell interactions. *Nature* **332,** 843–845 (1988).

96. Jiang, H. *et al.* T cell vaccination induces T cell receptor Vβ-specific Qa-1-restricted regulatory CD8+ T cells. *Proc. Natl. Acad. Sci.* **95,** 4533–4537 (1998).

97. Kim, H.-J., Verbinnen, B., Tang, X., Lu, L. & Cantor, H. Inhibition of follicular T-helper cells by CD8+ regulatory T cells is essential for self tolerance. *Nature* **467,** 328–332 (2010).

98. Leavenworth, J. W., Tang, X., Kim, H.-J., Wang, X. & Cantor, H. Amelioration of arthritis through mobilization of peptide-specific CD8+ regulatory T cells. *J. Clin. Invest.* **123,** 1382–1389 (2013).

99. Le Hir, H., Nott, A. & Moore, M. J. How introns influence and enhance eukaryotic gene expression. *Trends Biochem. Sci.* **28,** 215–220 (2003).

100. Hermel, E., Smith, C. & Aldrich, C. J. Allogeneic responses to the class Ib antigen Qa1: limited T-cell receptor Vα but not Vβ chain usage. *Immunogenetics* **51,** 600–605 (2000).

101. Brunner, K. T., Mauel, J., Cerottini, J.-C. & Chapuis, B. Quantitative assay of the lytic action of immune lymphoid cells of 51Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* **14,** 181–196 (1968).

102. Mbitikon-Kobo, F.-M., Bonneville, M., Sekaly, R.-P. & Trautmann, L. Ex vivo measurement of the cytotoxic capacity of human primary antigen-specific CD8 T cells. *J. Immunol. Methods* **375,** 252–257 (2012).

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Education

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GPA: 3.67 Major GPA: 3.74

Coursework: Molecular Biology and Genomics, Graduate Immunology, Pharmacology, Medical Entrepreneurship, Intermediate Microeconomics, Finance, Accounting, Economics & Business Statistics

Experience

Ph. D. Candidate/Fall 2010-Spring 2017
Johns Hopkins School of Medicine, Baltimore, Maryland
PI: Mark Soloski, Ph.D. – Division of Rheumatology

- Led research aimed at identifying biomarkers in several rheumatic diseases. Patient serum is profiled using high-throughput protein microarrays to identify candidate biomarkers. The discovery of these biomarkers has the potential for clinical applications that will aid a physician in the correct diagnosis and accurate prognosis.
- Pioneered implementation and data analysis for a novel microarray technology. Developed a metric to analyze 3.4 million data points in Excel.
- Manuscript published at the highest rated journal in rheumatology.

Equity Research Analyst - Externship/Winter 2016
T. Rowe Price, Baltimore, Maryland

- Health sciences focused buy side equity research analyst program. Analyzed the biotechnology sector to find undervalued investment opportunities. Developed models of market sizing, revenue forecasting, earnings per share estimates, and a discounted cash flow analysis to determine an appropriate valuation. Recommendations were contingent on a detailed pipeline analysis and assessing the likelihood of an FDA approval for new therapies.
- 1st place, stock pitch competition.

Research Technician II/Fall 2008 – Fall 2010
Duke University Medical Center, Durham, North Carolina
PI: Anne West, M.D., Ph.D./Geoffrey S. Pitt M.D., Ph.D. – Department of Neurobiology

- Developed a microscopy based imaging protocol to view and quantitate the number of inhibitory synapses in mouse models of learning and memory.
- Work produced publications in the Journal of Neuroscience and PNAS.

Genentech Externship/June 2008
Genentech, Inc., San Francisco, California

- Worked with the antibody engineering team in researching therapeutic antibodies.
- Improved molecular biology knowledge and techniques.

Finance Analyst/Summer 2007
Lehman Brothers Holdings Inc., New York, New York

- Provided a competitor analysis for the asset management industry, consolidated global expenses for the Investment Management Division, performed monthly expense analysis, published an internal newsletter, participated in finance training sessions, engaged in community outreach programs, and presented recommendations on an options trade capture system.

Leadership Activities

Johns Hopkins Biotech Investment Group – Executive Board Member

- Co-founder of a student run program aimed at educating Ph.D. students about the diversity of careers in biotech investing, and also developing the finance and economic skills to succeed in these positions. Focused on the areas of equity research, investment management, venture capital, and pharmaceutical management.

Johns Hopkins Consulting Club Biotech Case Competition – Finalist

- As part of a three member team provided market analysis for an innovative medical device designed at Hopkins. Determined the current market size for the product, projected revenue for years one through five, and identified how to best price the device.

Student Seminar Series Representative

- Organized the student invited seminar series for the Immunology Training Program. Responsibilities included selecting extramural speakers and coordinating their travel arrangements and interview schedule.

Varsity Men's Basketball

- 2006 and 2007 Centennial Conference Honor Roll – awarded to winter athletes who make a significant team contribution and maintain above a 3.4 GPA. 3-time varsity letter winner for a team that qualified for the Centennial Conference playoffs each year. Senior season was the winningest in school history.

Student Athlete Advisory Committee – Men's Basketball Representative

- Communicated with the faculty of Gettysburg College, conveyed the student-athlete's perspective on various administrative decisions, and fostered an excellent relationship between athletes and faculty.

Awards and Honors

American College of Rheumatology – Poster Presenter/2014

- Abstract on novel autoantibodies was selected for the American College of Rheumatology's poster presentation session.

Presidential Scholarship/2004-2008

- A merit based award presented by Gettysburg College to recognize excellent academic achievement during a student's high school career.

Arthur C. Musselman Award/2007-2008

- A merit based award funded by the Phi Delta Theta Educational foundation to a student embodying academic excellence and community involvement.

Publications

1. Pablo J., Wang C., **Presby M**., and Pitt G.S. Voltage-gated Na+ channels and FGF13/FGF14. *PNAS* (2016)
2. Fioretino D.F., **Presby M.**, *et al.* PUF60: a prominent new target of the autoimmune response in dermatomyositis and Sjögren's syndrome. *Annals of Rheum Dis.* (2015)
3. Erler, B.S., **Presby M.M**., *et al*. CD117, Ki-67, and p53 predict survival in neuroendocrine carcinomas, but not within the subgroup of small cell lung carcinoma. *Tumour Biol*. **1**, 107-111 (2011).
4. McDowell K.A., Hutchinson A.N., Wong-Goodrich, S.J., **Presby M.M.**, *et al*. Reduced cortical BDNF expression and aberrant memory in CaRF knock-out mice. *J. Neurosci*. **22** 7453-7465 (2010).